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Extracellular component hyaluronic acid and its receptor Hmmr are required for epicardial EMT during heart regeneration

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Abstract:	Aims: After injury, the adult zebrafish can regenerate the heart. This requires the activation of the endocardium and epicardium as well as the proliferation of preexisting cardiomyocytes to replace the lost tissue. However, the molecular mechanisms involved in this process are not completely resolved. In this work we aim to identify the proteins involved in zebrafish heart regeneration and to explore their function. Methods and Results: Using a proteomic approach, we identified Hyaluronan mediated motility receptor (Hmmr), a hyaluronic acid (HA) receptor, to be expressed following ventricular resection in zebrafish. Moreover, enzymes that produce HA, hyaluronic acid synthases (has), were also expressed following injury suggesting that this pathway may serve important functions in the regenerating heart. Indeed, suppression of HA production, as well as depletion of Hmmr blocked cardiac regeneration. Mechanistically, HA and Hmmr are required for epicardial cell epithelial-mesenchymal transition (EMT) and their subsequent migration into the regenerating ventricle. Furthermore, chemical inhibition of Focal Adhesion Kinase (FAK) or inhibition of Src kinases, downstream effectors of Hmmr, also prevented epicardial cell migration, implicating a HA/Hmmr/FAK/Src pathway in this process. In a rat model of myocardial infarction both HA and HMMR were upregulated and localized in the infarct area within the first few days following damage suggesting that this pathway may also play an important role in cardiac repair in mammals. Conclusions: HA and Hmmr are required for activated epicardial cell EMT and migration involving the FAK/Src pathway for proper heart regeneration.

CVR-2015-106

Extracellular component hyaluronic acid and its receptor Hmmr are required for epicardial EMT during heart regeneration

Maria A. Missinato, Kimimasa Tobita, Nicla Romano, James A. Carroll, and Michael Tsang

Abstract

Aims: After injury, the adult zebrafish can regenerate the heart. This requires the activation of the endocardium and epicardium as well as the proliferation of preexisting cardiomyocytes to replace the lost tissue. However, the molecular mechanisms involved in this process are not completely resolved. In this work we aim to identify the proteins involved in zebrafish heart regeneration and to explore their function.

Methods and Results: Using a proteomic approach, we identified Hyaluronan mediated motility receptor (Hmmr), a hyaluronic acid (HA) receptor, to be expressed following ventricular resection in zebrafish. Moreover, enzymes that produce HA, *hyaluronic acid synthases* (*has*), were also expressed following injury suggesting that this pathway may serve important functions in the regenerating heart. Indeed, suppression of HA production, as well as depletion of Hmmr blocked cardiac regeneration. Mechanistically, HA and Hmmr are required for epicardial cell epithelial-mesenchymal transition (EMT) and their subsequent migration into the regenerating ventricle. Furthermore, chemical inhibition of Focal Adhesion Kinase (FAK) or inhibition of Src kinases, downstream effectors of Hmmr, also prevented epicardial cell migration, implicating a HA/Hmmr/FAK/Src pathway in this process. In a rat model of myocardial infarction both HA and HMMR were upregulated and localized in the infarct area within the first few days following damage suggesting that this pathway may also play an important role in cardiac repair in mammals.

Conclusions: HA and Hmmr are required for activated epicardial cell EMT and migration involving the FAK/Src pathway for proper heart regeneration.

Keywords: Zebrafish heart regeneration, Hyaluronic acid, *hmmr*, epicardial cell migration, pFAK

Cover Letter/Declaration

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April 28th, 2015

Cardiovascular Research

Re: Resubmission of CVR-2015-106

Dear Editors:

Please find enclosed our resubmission of the manuscript entitled **Extracellular component hyaluronic acid and its receptor Hmmr are required for epicardial EMT during heart regeneration",** which we would like to be further considered for publication in ***"Cardiovascular Research"***.

We like to thank the reviewers and editors for taking the time in critically reviewing this work. In this resubmission we have changed the title at the request of the reviewer to be consistent with the use of Hyaluronic Acid instead of interchanging with Hyaluronan. Hence the title is changed to use Hyaluronic Acid. We have also addressed all the concerns that the reviewers and provided a point-by-point answer to their comments. The revised manuscript is highlighted in yellow to reflect changes in the text. In addition, we have not included new experimental data to the main manuscript as well as to the supplementary materials.

This study describes a proteomic approach to identify factors that are up-regulated following cardiac injury in zebrafish. The adult zebrafish can regenerate its heart after injury and provides a model to study cardiac repair. We identified Hyaluronan mediated motility receptor (Hmmr), a hyaluronic acid (HA) receptor, to be increased following injury and our studies show that this protein plays an important role in heart regeneration. Mechanistic studies reveal that HA is required in epicardial cells following injury in order to undergo epithelial-to-mesenchymal transition. This process is critical for activated epicardial cells to migrate into the injury site and promote neovascularization of the regenerating heart. Moreover, we demonstrate that phosphorylation of Focal Adhesion Kinase (FAK) in epicardial cells is decreased after Hmmr depletion or when HA production is suppressed. In addition we demonstrate that Src Kinases are downstream of Hmmr/FAK as suppressing Srcs with chemical inhibitors exhibit similar phenotype. Thus we have revealed a signaling mechanism where HA and its receptor, Hmmr are required for epicardial cell migration through FAK and Src. To demonstrate the potential importance of this pathway in mammals we reveal that Hmmr and HA are also upregulated in rat hearts after myocardial infarction.

Of course, upon acceptance, I, the corresponding author of this manuscript will be willing and responsible for all the colour charges associated with the publication of this work.

We believe that this study would be of interest to readers of ***Cardiovascular Research*** and thank you for taking to time to consider this submission. We look forward to hearing from you soon.

Sincerely,

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Dear Editors and reviewers,

We thank the reviewers and editors for taking the time to critically review our study. We appreciate the constructive criticisms to our manuscript and have now addressed all of the concerns and comments over the past few months. We have now included new experiments and text changes as suggested and are thankful that this has improved this study and our conclusions that Hyaluronic Acid plays a critical role in cardiac regeneration. We have provided below an explanation as to how we have addressed each concern (in blue).

Reviewer #1: The manuscript by Missinato et al. reports a series of experiments pointing to an important role for hyaluronic acid synthesis and the hyaluronan mediated motility receptor (Hmmr) in the epicardial cell migration during heart regeneration in zebrafish. They also present data indicating involvement of FAK and Src signalling as Hmmr downstream effectors. The topic and experimental approach are interesting, but the results and conclusions need to be strengthened. The following points need to be addressed:

It is important to provide the full names and briefly mention the function of the genes identified as differentially expressed during heart regeneration. Also Has should be mentioned as hyaluronic acid synthase (Result session, first paragraph).

We changed the text adding the full names of the genes. The function of the genes found up-regulated during heart regeneration is indicated in the first paragraph of the discussion.

In Fig.1A and 1C a box defining the clot area should be added. In Fig1C a color code of the AFOG is useful. Moreover, a definition of the damaged area with dashed lines should be added. In the immunofluorescence corresponding to 7dpa the staining for HA, in red, seems to be diffused in other regions of the heart, while at 3dpa and 14 dpa the staining seems to be restricted to the clot area. Can the author clarify whether this is an event that consistently occurs at 7dpa in the whole heart or just an effect of the staining in the specific panel?

We changed Figure 1 to show the clot area. The color code for AFOG is described in the Fig 1 legend: “AFOG stains intact cardiac muscle in yellow-orange, fibrin in red, and collagen in blue.”
We add in the text that HA at 3 and 7dpa is in the clot, and in the border of damage-not damage tissue. The localization of HA appears to be consistently in the clot tissue. In some hearts at 7dpa we do observe it within the cardiac tissue adjacent to the injury site as the reviewer has noted. We are not sure why we detect this in some instances but not always.

In Fig.2 the indication of the dpa should be added above each of the panels, a box of the

clot region should also be present in panels F-G. The analysis of Fig2 I-J should be extended also at the 30dpa stage or, if already performed, the result of this experiment should be at least added in the text.

We changed Figure 2 indicating dpa, and a box showing the clot region. We add the immunostaining for alpha-SMA and MHC at 60dpa (10d injections with 500HMC) (Fig S3), showing that myofibroblasts are still present in hearts injected with HMC.

In Fig3 the experiments are performed at 10dpa, while in Suppl. Fig S3 at 7dpa and make them difficult to compare. It might be interesting to assess whether the defective migration of cardiomyocytes is an early event or does not occur at all (so very few or no cardiomyocytes might be present even at 30dpa). Moreover, a marker of neovascularization should be included in the analysis (i.e.VEGFR2)

Fig S3 showed heart sections at 7dpa because we assessed cardiomyocyte proliferation at a point when this occurs at its peak. Fig3 figures was performed at 10dpa because we wanted to measure the new vessels being formed, which initiates around this time in ventricular resection experiments. To confirm the lack of cardiomyocytes at later time points we immunostained for Mef2c and MHC at 60dpa (Fig S5). As shown in Fig. S5 we did not detect any cardiomyocytes in hearts injected with HMC, suggesting that in absence of HA cardiomyocytes do not populate the injured area. We also added experiments to demonstrate that angiogenesis is affected after HMC injections. We used Fli1b antibodies to mark endothelial nuclei in injured hearts at 10dpa and showed the number of endothelial cells in the clot tissue was significantly reduced after HMC treatment. This is now included in Figure 3 (Fig. 3I-K).

In Fig.4 panels A-D a box indicating the analyzed scar tissue should be included. In Fig.S5B a RT-PCR showing the different products originating in presence or absence of the different treatments (especially VMO and mutVMO) should be included with a marker indicating the approximate size of the products.

We changed Fig 4, adding a box to show the scar area. We changed Fig S5 (NOW Fig S7) showing the MW marker in the PCR products.

In Supplementary Fig.S6 the hmmrVMO seems to induce an increase in the percentage of MHC positive cells in the whole heart and at least in some regions (other than the clot area) the hmmrVMO seems to promote a stronger angiogenesis induction compared to the other conditions. Can the authors make a comment regarding this aspect?

In Fig S6 (NOW Fig S8) the reviewer is correct in pointing out that MHC staining is stronger when compared to the controls (PBS & *MutMO*). The reason for this was that the exposure was turned up to ensure that there were no weak GFP cells in the clot, further highlighting that *hmmrVMO* suppressed angiogenesis. This is also the reason why overall GFP fluorescence is stronger in the myocardium.

In Fig.5 the panels A-D reports an experiment performed at 5dpa, while the scatter plot in

E refers to 7dpa and the western blot in F reports the activation of pFAK after 1dpa, so that it's hard to compare them.

We apologize for the confusion caused with Figure 5 and the multiple time points used to monitor pFAK. In Figure 5A-D, we wanted to show both the lack of wt1EGFP+ cells that were pFAK staining in the clot tissue at 5dpa. Since epicardial cell migration spans several days we wanted to confirm that even at 7dpa there was a reduction of Wt1+EGFP cells in the clot tissue and that HMC injections did not just delay epicardial cell EMT and migration. The western blot was performed at 1dpa to show that even at the earliest time point, pFAK was generally decreased after HMC injections.

Panels G,H,K,L, show results from 3dpa hearts (similarly to what observed for the Fig2 B-E) when also the QPCR analysis on EMT markers is performed, but it's not specified if the region is the clot area or another one. If the region is the clot area the number of activated epicardial cells at 3dpa seems to be very similar in PBS and HMC treated hearts, while at 5dpa the number of wt1bEGFP positive cells is decreased in HMC, compared to PBS control group. So if there is a defect in epicardial cells migration that depends on pFAK activation, it should be earlier than 5dpa, since already at 1dpa the western blot already shows that HMC blocks pFAK activation. Can the authors provide an explanation, even just in the discussion section?

These panels (F, G, J, K, L) show areas of the epicardium and we have added this to the Figure 5 legend: "(**F, G**) Confocal images of activated wt1b^{+} cells at the epicardial region at 3 dpa after injection of control PBS (**F**; n=4) or HMC (**G**; n=5)". This region shows similar number of wt1bEGFP^{+} cells between the various treatments. However pFAK and cell morphology appears to be different. As shown in Figure 5F & 5G, the general Wt1bEGFP^{+} cell shape in controls are more round (5F) as compared to HMC injected hearts (5G). We have added this conclusion to the discussion section.

As concerning the different cell morphology evidenced in panels G,H,K,L in presence of HMC, can the author find a quantitative method to count the percentage of elongated vs rounded cells? Moreover, would it be possible to see the effect of the hmmrMUTVMO at 3dpa, as for panel L?

We have now added the quantification of Long vs Round epicardial cells (Fig S10F) after HMC treatment. We add a new panel to show the phenotype of Wt1bEGFP^{+} cells in hmmr-MutMO injected hearts (Fig 5J-K and L).

In Fig. 6 (panels in A,B,C) it is not indicated for how long the inhibitors were injected. In the materials and methods it is stated that "the injections were performed daily for up to 10 days at which points the hearts were extracted".

We have added to the figure legends to indicate that inhibitors were injected daily from day 1 to day 10pa.

In panels from H to M it would be desirable to add the name of the treatment in the lower left corner of the immunofluorescences.

We have added the names of the treatments in panels in Figure 6.

In Fig7 the experiment presented in panels A and B should be performed also in presence of the hmmr MUTVMO.

We have added the *hmmr-MutMO* data to show that migration of epicardial cells in the ex vivo assay is comparable to other controls and significantly different than *hmmr-VMO* (Fig 7F).

To asses the importance of HA during cardiac regeneration the authors use 7-Hydroxy-4-methylcoumarin to inhibit Has activity, but do not to show a dose response effect or the actual in vivo inhibition of the enzymatic activity.

We have added a new panel to a figure to show the dose response of HMC (New Fig S2B). It is clear that lower doses (3ul of 50 and 100µM concentration injected), HA is still present in the clot tissue of injected hearts. We used 3µl of 500µMHA as the dose for all the experiments reported in this study.

The inhibition of epicardial cell migration as well as the inhibition of angiogenesis should be measured by semi-quantitative method (cell/area).

We quantified new vessels/clot area (Fig 3H and G) and epicardial cells/clot area (Fig 5E), but in the ex-vivo migration experiments (Figure. 7) it is difficult to accurately count the number of cells because they are in different planes.

English needs checking from mother language person. (i.e. in the 5th line of the introduction: ?although some evidence?might be followed by "indicates that", while in the 3rd line from the bottom of the introduction section, the word "decrease" should be substituted with "decreased").

We checked the English and changed those sentences as suggested by reviewers.

Reviewer #3: General comments:
The paper nicely describes the role of hyaluran and one of its receptors in cardiac regeneration after injury in the zebrafish.

Specific comments:
How specific is Hydroxy-4-methylcoumarin (HMC)? This really neeeds to be documented as it is crucial to the interpretation of the data.

There are several references using HMC (also known as 4-MU); we reported only one reference in keeping with the limited space and reference count of the journal. We have

now added another reference that further supports the specificity of HMC on Has2. In the text we include the statement “HMC inhibits HAS by depletion of the cellular substrate, UDP-glucoronic acid”.

What is the interaction of the HA-HMMR pathway with the other pathways involved in EMT and migration such as FGF, TGF<beta>, PDGF<beta> 14, IGF2, RA, Jak1/Stat3 and Notch signaling? This can be investigated by investigating their expression during the inhibition experiments.

This is an interesting avenue of study, but we feel that this is beyond the scope of the current work. This also depend the time and stage after injury and so would take another year or two in order to properly conduct these experiments.

What is the role of CD44 and TLR and how does the HA-HMMR pathway interact/influence these receptors as CD44 expression also increases (fig 1)?

As with the previous comment we feel that these HA receptors may play an important role in heart regeneration, but in order to studies these it would take some genetic or knockdown approaches. This is an extensive undertaking.

The rat MI data should be mentioned in the abstract.

We have added the rat data in the abstract.

The selection of HMMR is based on Differential gel electrophoresis which was only performed at day 3 after. Why was this point selected? Why did the authors not perform a time course after amputation as was done for the RT-PCR?

We did 2D-gel analysis at 3dpa because we knew from the literature that this is a crucial time point for zebrafish heart regeneration. From this pilot study we found Hmmr to be up-regulated at 3dpa, so then we decided to confirm the proteomic results and to uncover the function of Hmmr in heart regeneration. Since there have been limited studies on Hmmr, we felt that its function in heart regeneration could be somewhat novel. To perform a time course analysis with DIGE is much more expensive and time consuming than to do qPCR. We do not have the resources to pursue what the reviewer suggested.

Hmmr expression was markedly increased throughout the whole heart (Fig. 1A). Is that not surprising? I would expect an increase around the injury site comparable to HA (Fig. 1C)

It has been reported already that zebrafish heart respond to injury with a re-expression of many genes throughout the entire heart (Atrium, ventricle and BA), and then, with the time, the expression became restricted to the injury area. So it is not unprecedented that the whole heart responds to injury.

The data on HA content (fig 1 and 2) need to be quantified as was done for the data on ASMA (fig 2H).

We have not included quantification of HA content in Fig2 and this is now in Supplementary Fig 2.

HMC reduces the number of cardiomyocytes inside the clot, while no change in PCNA was found at day 7 (Fig. 3A-B and G). The authors implicate a deficiency in cardiomyocyte migration, yet there may also be a shift in the proliferation peak and height and even cardiomyocyte cell death. Therefore this remark needs to be altered or substantiated.

We have added the remark that cadiomyocytes failed to repopulate the clot tissue because they fail to migrate or that after the proliferative phase, CM survival is affected in the absence of HA.

The authors now mention that the HMC reduces epicardial EMT, pFAK/SRC and subsequent endothelial and cardiomyocyte migration. That HM affacts both endothelial and cardiomyocyte migration is interesting yet also evokes several questions, such as which effect is more important for cardiac regeneratio, how does suppression of angiogenesis affect CM migration? Are the molecular pathways involved really identical, what is the role of other pathways and HA receptors (see above)?

Cardiac regeneration requires the orchestration of many cell types and events following injury. The pathways involved, like in development could be reiteratively used for several steps in heart regeneration. Thus altering one could affect many independent events that lead to a failure of regeneration. An example of this is the role of PDGF signaling in epicardial cell EMT and angiogenesis (Kim et al. PNAS 107:17206-10.)

The rat data are quite superficial as only expression data are shown. The authors should provide data on an HMC inhibition experiment in the rat MI model.

Previous studies have showed that introducing HA into a rat infarct model can improve cardiac function and limit scar tissue. This study is cited in the discussion section. Our data in the rat shows that upon injury, HA and HMMR is induced suggesting that the response to cardiac injury in zebrafish is also conserved in mammals.

The first paragraph of the discussion can be deleted as no data on Desmuslin are shown.

There is conflict with reviewers on whether this data on Desmuslin should be included. We have included this and would not object if the editors decide to remove this part of the discussion.

The discussion is very short and superficial. Limitations of the studies are not discussed, no real new insights are provided. Therefore this part needs to be rewritten.

We changed the discussion accordingly including limitations and new insights.

Reviewer #4: In this study, Missinato et al investigate the role of hyaluronic acid (HA) and its receptor, Hmmr, in cardiac regeneration in the Zebrafish. The authors find that both HA and Hmmr are required for proper regeneration and neo-angiogenesis through FAK and Src.

This is an interesting and mechanistic study. In its present form, however, the manuscript presents major limitations.
1. The manuscript is difficult to read. It is hard to follow the story, especially in the Results section. Extensive rewriting and careful grammatical proofreading will be required to eliminate meaningless sentences. Examples: "Cardiomyocytes (Mef2C)that were in S phase (PCNA) was not statistically significant?"; "?were retro-orbital injected"; "to measure expression of EMT genes"; "interpreted as increasing of energy demand", etc?

We have corrected the text as suggested by the reviewer.

2. The Discussion is a mere summary of the results. It should also be rewritten to put the data in perspective of other advances in the field

We have made changes to the discussion as suggested.

3. The title mentions hyaluronan whereas the remaining of the manuscript talks about hyaluronic acid. Consistency is necessary.

We changed the text and now we use only “Hyaluronic Acid” instead of “Hyaluronan”.

4. Both the title and the conclusion of the abstract imply that cardiac regeneration is the result of EMT. This cause-consequence relationship is not demonstrated in the manuscript. Rather, as mentioned in the Introduction, a "key factor of regeneration" is proliferation, whereas, "Another feature" is the activation of EMT for angiogenesis.

It is clear that both epicardial cell activation and EMT along with CM proliferation are both required for proper heart regeneration. In addition the activation of the endocardium is also important and that the activities of the whole heart in response to injury is required for proper regeneration. We have changed to text to reflect this.

5. Methods. Several transgenic lines are mentioned in the text, but it is nowhere mentioned why they were used or for what purpose. All the sudden, these lines appear in the Figures without any explanation.

We have now added in the text an explanation of the transgenic lines and the rational for their use.

6. Figure 2. The changes in abundance of HA should be shown in a more quantitative way, by western blotting for example.

HA is not a protein and cannot be detected by western blotting. We have used the best available approach that is to detect HA using a biotin-conjugated HA binding protein (HABP). We have quantified this as labeling of HABP and show this in Supplementary Figure S2C.

7. Figure 2A is not necessary.

We believe that the cartoon in Fig 2A makes easier for the reader to understand how we did the chemical treatments. We will leave it up to the editors to determine if this should be removed.

8. Figure 5I. Is there any statistical significance among groups?
We have repeated a few more qPCR experiments to determine if gene expression changes are significant. In this analysis, statistical difference for snail 1b was observed, so now we adjusted the figure as suggested by the reviewer (now Fig 5H). Expression for snail 1a, snail2 and twist1a were not significantly different, however the general trend was decreased expression in the three experiments.

9. Figure 5F and 5J. These panels should offer a quantitative analysis from several samples rather than one qualitative example, which we have to believe being representative.

We have moved the western blot of pFAK to supplementary Figure S10E. This is a representative western of 3 experiments. With respect to Twist western analysis we have repeated four times and overall Twist expression is decreased in three experiments but was not changed in one experiment. Because injury and regenerative response is not uniform, we expect some variation from experiment to experiment.

10. Figure 6. The legend mentions the use of a Student's t test, whereas it should be a multi-group ANOVA.

The reviewer is correct and we have changed Figure 6 legend, stating that one-way ANOVA was used in the statistical analysis.

11. The authors should do a better job at presenting, analyzing and interpreting the DiGE and MS/MS analysis. How many spots were found to be different? How many spots were cut out and identified? Provide a list of all these proteins. On what criteria was based the characterization of three proteins, specifically?

We add this information in the text to state that this was a pilot proteomics to demonstrate feasibility. We only picked a handful of protein spots as the LC/MS could not identify all the spots due to weak signal. The limitation of this approach is that DIGE is very sensitive that is beyond the level of detection of standing LC/MS.

Editorial assessment

In this study, the authors investigate the role of hyaluronic acid (HA) and its receptor, Hmmr, in Zebrafish cardiac regeneration. The authors find that both HA and Hmmr are required for proper regeneration and neo-angiogenesis through FAK and Src. Although the study is interesting and mechanistic, in its present form, however, the manuscript presents major limitations related to the data presentation and interpretation. In addition, the authors should provide evidences that their findings could be relevant in other animal/models than zebrafish.

We found that the HA pathway was conserved in rat and at least one study has shown that treating rat heats with HA can improve cardiac function. However the mechanism as to the role of HA is unclear. Here we provide data to suggest that HA is required for cardiac regeneration through regulating epicardial EMT.

Checklist

- Highlight in the manuscript all changes that have been made;
- Mention in the cover letter all changes in the title and / or authors and repeat all necessary declarations as stated in our instructions to authors;
- Use the manuscript number CVR-XXXX in the running head of every page.

CVR-2015-106

Extracellular component hyaluronic acid and its receptor Hmmr are required for epicardial EMT during heart regeneration

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Maria A. Missinato, Kimimasa Tobita, Nicla Romano, James A. Carroll, and Michael Tsang

Abstract

Aims: After injury, the adult zebrafish can regenerate the heart. This requires the activation of the endocardium and epicardium as well as the proliferation of preexisting cardiomyocytes to replace the lost tissue. However, the molecular mechanisms involved in this process are not completely resolved. In this work we aim to identify the proteins involved in zebrafish heart regeneration and to explore their function.

Methods and Results: Using a proteomic approach, we identified Hyaluronan mediated motility receptor (Hmmr), a hyaluronic acid (HA) receptor, to be expressed following ventricular resection in zebrafish. Moreover, enzymes that produce HA, *hyaluronic acid synthases* (*has*), were also expressed following injury suggesting that this pathway may serve important functions in the regenerating heart. Indeed, suppression of HA production, as well as depletion of Hmmr blocked cardiac regeneration. Mechanistically, HA and Hmmr are required for epicardial cell epithelial-mesenchymal transition (EMT) and their subsequent migration into the regenerating ventricle. Furthermore, chemical inhibition of Focal Adhesion Kinase (FAK) or inhibition of Src kinases, downstream effectors of Hmmr, also prevented epicardial cell migration, implicating a HA/Hmmr/FAK/Src pathway in this process. In a rat model of myocardial infarction both HA and HMMR were upregulated and localized in the infarct area within the first few days following damage suggesting that this pathway may also play an important role in cardiac repair in mammals.

Conclusions: HA and Hmmr are required for activated epicardial cell EMT and migration involving the FAK/Src pathway for proper heart regeneration.

Keywords: Zebrafish heart regeneration, Hyaluronic acid, *hmmr*, epicardial cell migration, pFAK

1. Introduction

Ischemic heart disease is one of the most common causes of mortality in developed countries. After myocardial infarction (MI), billions of cardiomyocytes undergo apoptosis, pyroptosis, and necrosis and a non-contractile collagen scar that limits the cardiac function is formed (*1*). The ability of the mammalian heart to replace lost cardiomyocytes is limited (*2*). Neonatal mice are able to regenerate after amputation of the ventricular apex (*3*), and after MI (*4*), but regeneration is restricted to post-natal day 7 (P7), after which the majority of cardiomyocytes become post-mitotic. More recently, cardiac ischemic injury during the phase of cardiac growth in adolescent mice (P15) also exhibited regenerative capacity, suggesting that under certain conditions regeneration can occur in mammals (*5*). In contrast, adult zebrafish (*Danio rerio*) can efficiently regenerate the heart after amputation of the ventricle apex throughout its lifespan (*6, 7*). A key factor of the regenerative process in zebrafish is the ability of pre-existing cardiomyocytes to undergo proliferation following organ damage (*8-11*). Another feature is the activation and proliferation of epicardial cells to undergo epithelial-mesenchymal transition (EMT), followed by their migration into the injury site to promote angiogenesis (*9, 12-14*). In addition, within 3 hours of ventricular injury, the endocardium undergoes morphological changes and induces the expression of retinoic acid (RA)-synthesizing enzyme *aldh1a2*, indicating that the endocardium is also a dynamic player in zebrafish heart regeneration (*13*). The molecular pathways that direct these processes are beginning to be elucidated with evidence that Fibroblast Growth Factors (FGF) (*11, 14*), Transforming Growth Factor-β (TGFβ) (*15*), Platelet Derived Growth Factor β (PDGFβ) (*16*), Insulin like Growth Factor 2 (IGF2) (*17*), RA (*13*), Jak1/Stat3 (*18*) and Notch signaling (*19*) playing important roles to promote cardiomyocyte proliferation, endocardium activation and epicardial EMT.

In this pilot study we analyzed the proteomic changes following cardiac resection of the ventricular apex in adult zebrafish. We identified increased expression of an Hyaluronic acid receptor (Hmmr) and hypothesized that the Hyaluronic Acid (HA) pathway could play a crucial role in cardiac regeneration. HA is a large, linear non-sulfated glycosaminoglycan component of extracellular matrix (ECM). Following injury HA is produced in the inner side of the plasma membrane by Hyaluronic Acid Synthases (HAS), and is extruded onto the cell surface where it accumulates in the wound to promote cellular proliferation, and migration to support tissue remodeling and healing (*20*), (*21*). Chemical suppression of HA synthesis as well as knockdown of Hmmr blocked cardiac regeneration. Mechanistically, we observed decreased migration of activated epicardial cells into the regenerating heart and reduced coronary vasculature, suggesting that HA is important for epicardial epithelial-to-mesenchymal transition. Our studies document the importance of HA and its receptor in epicardial cell migration into the clot tissue for remodeling of the nascent coronary vasculature.

2. Methods

2.1 Zebrafish maintenance, ventricular amputation and retro-orbital injections

The zebrafish experiments were performed according to protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh. Adult (6-18 months) wild type AB* and transgenic *Tg(myl7:EGFP)*^{f1} *(22)*, *Tg(fli1a:EGFP)*^{y1} (*23*), *Tg(wt1b:EGFP)*^{li1} (a kind gift from Christoph Englert) (*24*) zebrafish were maintained at 28°C. Zebrafish were anaesthesized by immersing in 0.168g/L Ethyl 3-aminobenzoate methanesulfonate salt (MS-222; Sigma) for 3-5 minutes. Zerbafish were placed onto a wet sponge with the abdomen facing up.

Approximately 20% of the ventricle apex was resected as described by (*6*), and zebrafish returned to the system for recovery before retro-orbital injections of drugs or with *Vivo Morpholinos (VMO)* at one day post surgery as described by (*25*). Retro-orbital injections were performed daily for 2, 4, 6, or 10 days at which point hearts were extracted. To suppress HA production, zebrafish were injected with 3 µl of 500 µM 7-Hydroxy-4-methylcoumarin (HMC) (Acros Organics) (also known as 4-methylumbelliferone, 4-Mu), or with PBS as vehicle control. To inhibit FAK, 3 µl of 100 µM of PF-573228 (Sigma) dissolved in DMSO (Sigma) were injected. To suppress Src Kinase, 3 µl of 200 µM Src Inhibitor 1 (SKI-1) (Sigma) or vehicle DMSO were injected. To knockdown Hmmr, 1 µl of 3 mg/Kg Hmmr E414 spliced *VMO* (Gene Tools, LLC) (TGTGCAAACAGATGTACCTCTTTCT) was injected. For controls, 5 bp mismatch *VMO* (Mut-MO) (TGAGGAAACACATCTACGTCTTTCT) was retro-orbitally injected.

2.2 Difference gel electrophoresis (DiGE) and MS/MS analysis

A detailed description of DiGE and MS/MS analysis is provided in the Supplementary Material.

2.3 In situ hybridization (ISH), immunostaining, clot area measurement and cell counting

For histological examination, zebrafish were euthanized using 0.168g/L Ethyl 3-aminobenzoate methanesulfonate salt for 15 mins and hearts were collected in cold PBS and fixed in 4% paraformaldehyde (PFA) overnight at 4°C. Hearts were cryopreserved with sucrose before immersion in embedding media (Instrumedics). 14 µm cryosections were collected and consecutive sections were used for ISH, immunostaining and Acid Fuchsin Orange G (AFOG). ISH was performed in cryosections, using digoxygenin-labeled cRNA probes as described (*14*). BM purple (Roche) was used as alkaline phosphatase substrate. AFOG staining was performed as described by (*6*). Images were captured with Leica MZ 16 microscope and Q Imaging Retiga 1300 camera. A detailed description of clot area measurement and cell counting is provided in Supplementary Material.

2.4 RNA extraction, cDNA synthesis, PCR, and quantitative PCR (Q-PCR)

Total RNA was isolated from uninjured hearts and hearts at 1, 3, and 7 days post amputation (dpa) using TRIzol reagent (Invitrogen), and RNeasy Micro kit (Qiagen), according to manufacturer's instructions. 8 hearts were pooled together for each condition. 1 µg of total RNA was reversed transcribed to cDNA with SuperScript (Invitrogen) using random hexamers. PCR was performed to test the efficacy of *hmmr* Spliced V-MO knockdown. *Eukaryotic translation elongation factor 1 alpha 1, like 1* (*eef1a1l1*) was used as reference gene. The primers sequences for PCR are the following: *eef1a1l1*-F ATCTACAAATGCGGTGGAAT; *eef1a1l1*-R ATACCAGCCTCAAACTCACC; *hmmr*-Ex3-F GGACCATGTCTGTTGATGGTTTGGCTG; *hmmr*-Ex-7-R GACCTTTACCTTTCCTTCTGAGC. RT-PCR products were electrophoresed on agarose gels and stained with ethidium bromide. The primers sequences used for Q-PCR were designed using Beacon designer and are listed in Table 1 in Supplementary Material. Two step real time PCR was performed using SYBR Green (Biorad) in a PCR system iQ5 thermal cycler (Biorad). Primers set efficiency was calculated and adjusted using LinRegPCR. *ß-actin* and *RNA polymerase* were used to normalize gene expression in the Q-PCR experiments. Experiments were done in triplicate.

2.5 Western Blot

Proteins were extracted from a pool of 5 ventricles apex of each condition, using 200 µl of Laemmli Buffer (Bio-rad), containing ß-mercaptoethanol (Fisher). Samples were heated at 95ºC for 5 minutes, and 0.1 M dithiothreitol (Calbiochem) was added before loading. After SDS-PAGE, proteins were transferred in nitrocellulose membrane (Li-cor). Membrane was blocked in Odyssey Blocking Buffer (Li-cor) for 1 hour. Antibodies were diluted in Odyssey Blocking Buffer, containing 0.2% Tween 20 (National diagnostics). Blots were scanned using Li-cor Odyssey CLx Infrared imaging system, and band intensity were quantified and normalized using Image Studio software. Primary antibody used: anti-Twist1 (Sigma) (1:1000), anti-ERK-2 (Sigma) (1:500), anti-actin (Sigma) (1:5000), anti-FAK [pY397] (Invitrogen) (1:1000). Secondary antibody used for western blot were: IRDye 800 donkey anti-rabbit IgG (H+L) (Li-cor) (1:15000), and IRDye 680 goat anti mouse (H+L) (Li-cor).

2.6 *Ex vivo* Cell Migration Assay

Epicardial cell migration was measured in *ex vivo* assay as described by (*26*), using *Tg(wt1b:EGFP)*^{li1} fish. Briefly, 3 dpa hearts were extracted from zebrafish retro-orbital injected for 2 days with HMC, *hmmr VMO*, PF-573228, SKI-1, or vehicles PBS and DMSO. Hearts were cultured in 24-well plate pre-coated with fibrin and incubated at 28°C for 3 days. Cell migration was measured using ImageJ from the edge of the heart to the edge of the cell monolayer at 1, 2, and 3 days post extraction.

2.7 Rat maintenance and Myocardial Infarct (MI) induction

A detailed description of rat maintenance and MI induction is described in Supplementary Material.

2.8 Statistical analysis

Statistical significance was analyzed by the Student's *t*-test, One-way ANOVA and 2way ANOVA and shown as mean ± standard deviation. P-values were considered significant when < 0.05.

3. Results

3.1 Atp5a1, Desmuslin and Hmmr are up-regulated after heart injury

To identify the proteins that are differentially expressed during zebrafish heart regeneration we performed a pilot DiGE experiment on 3 days post amputation (dpa) and control uninjured hearts (fig. S1A-B). We picked the most prominent protein spots that were within the detection range of MALDI-TOF/TOF-MS for protein identification. We identified ATP Synthase 5a1 (Atp5a1), Desmuslin, and Hyaluronan mediated motility receptor (Hmmr), to be increased in heart samples at 3 dpa (fig. S1A-C). Previous work revealed the importance of HA in cardiac development and tail regeneration (*27*, *28*). However, the role for HA receptor in regeneration is not known. Therefore, we investigated the function of Hmmr in zebrafish cardiac regeneration. We verified *hmmr* gene transcription was also increased following injury by *in situ* hybridization (ISH) and Q-PCR. *hmmr* expression was absent in uninjured hearts but was markedly increased throughout the whole heart (Fig. 1A) and confirmed by Q-PCR (Fig. 1B). A rapid increase in *hmmr* expression was observed at 3 dpa, but expression declined at 7 dpa (Fig. 1B). Similarly, expression of *cluster of differentiation-44 (cd44)*, a co-receptor for HA, was induced at 3- and 7 dpa. Moreover, the enzymes that synthesize HA, Hyaluronic Acid Synthases (*has1* and *has2)* were also upregulated at 3 dpa (Fig. 1B). In contrast, expression of *hyaluronidases2 (hyal2)*, an

enzyme responsible for HA degradation, did not change, suggesting that upon injury HA accumulates in the heart.

To confirm the presence of HA in the heart, we used biotinylated HA binding protein (bHABP) to detect HA accumulation following cardiac injury (*29*). In uninjured hearts, HA was not detectable, but at 3- and 7 dpa substantial HA was localized in cells adjacent to the wound and also within the clot (Fig. 1C). By 14- and 30 dpa the HA was still present, but was restricted to the clot tissue (Fig. 1C). Furthermore, immunostaining for Has proteins revealed that expression was restricted in the clot area and co-localized with HA, confirming that upon injury, HA accumulates within the damaged tissue (fig. S2A) (*30*). Thus Has proteins and HA receptors are rapidly induced following cardiac damage suggesting that these factors are important in zebrafish heart regeneration.

3.2 HA is required for proper heart regeneration

To determine the importance of HA during cardiac regeneration, we suppressed HA synthesis after ventricular resection using 7-Hydroxy-4-methylcoumarin (HMC), an inhibitor of Has enzymes that depletes the cellular substrate, UDP-glucoronic acid (*31*, *32*). HMC was delivered by retro-orbital injections following cardiac injury (Fig. 2A). At 3 dpa, HA in the clot area was greatly diminished in HMC injected fish (Fig. 2B-E, fig. S2B & C). By 30 dpa, when regeneration is complete (*6*) (Fig. 2F, H), HMC-injected zebrafish still contained significant scar tissue (Fig. 2G, H). In concordance with these observations, at 15 dpa, control zebrafish (PBS injected) α-smooth muscle actin (α-SMA), a marker for myofibroblasts and damage was not observed (Fig. 2I). However, in HMC injected zebrafish, a strong presence of α-SMA, especially within the injured apex, was present (Fig. 2J & S3), indicating that activated fibroblasts are still present in the clot area.

To understand mechanistically how HA may function in cardiac regeneration we measured cardiomyocyte proliferation in HMC injected adults. At 7 dpa, cardiomyocytes (Mef2c) that were in S-phase (PCNA) was not statistically different from control PBS-injected zebrafish (fig. S4). However at 10 dpa, a statistically significant reduction in cardiomyocytes inside the clot was observed as compared to controls, implicating a deficiency in cardiomyocyte repopulating the clot tissue (Fig. 3A-B and G) (*10*, *33*). This lack of cardiomyoctye in the wound was observed even at 60 dpa (fig. S5) in HMC injected zebrafish. This lack of cardiomyocytes in the clot tissue could reflect a migratory defect and their survival in HMC injected hearts after the proliferative phase. Another important aspect of cardiac regeneration is the establishment of the coronary vasculature to support the regenerating cardiac tissue. We next determined if the formation of the coronary vasculature was also disrupted. HMC injections into a transgenic line that labels blood vessels with green fluorescent protein (*Tg(fli1a:EGFP)*), showed a significantly reduction in coronary vasculature in the clot area (Fig. 3C-F and H, and fig. S6). Moreover, the endothelial cells that were detected in HMC injected zebrafish were sparse and failed to organize into vessel-like structures. The lack of coronary vasculature was confirmed by immunostaining for the presence of Fli1b, another endothelial expressed Ets-transcription factor important for vessel formation (*34*). In HMC injected zebrafish, there was a pronounced decrease in $Fli1b^{+}$ cells within the clot (Fig. I, J and K). Taken together, these results show that HA is produced following cardiac injury and plays a critical role in the formation of the coronary vasculature and in the repopulation of cardiomyocytes in the clot tissue.

3.3 *hmmr* is necessary for heart regeneration

Given the importance of HA in heart regeneration, we next determined if the Hmmr is also required in this process. *hmmr* was knocked-down using *Vivo-Morpholinos* (*VMO*). *VMOs* have been shown to be effective in targeting the depletion of protein expression in several tissues in mice and in adult zebrafish (*35*). The *hmmrVMO* was designed to suppress intron splicing between exons 4 and 5 (fig. S7A). RT-PCR experiments confirmed that *hmmr* transcripts in pooled injured hearts were reduced in zebrafish injected with *hmmrVMO,* but not in control *hmmr-MutMO* (5-bp mismatch *vivo* morpholino) (fig. S7B). Following ventricular resection, *hmmrVMO*, *hmmr-MutMO*, or PBS were retro-orbital injected and at 30 dpa the hearts were extracted for analyses. Scar tissue area was significantly larger in *hmmrVMO*-injected zebrafish as compared to PBS and to the *hmmr-MutMO* controls (Fig. 4A-D). As noted with suppressing HA production, knockdown of *hmmr* also decreased angiogenesis at 10 dpa (Fig. 4E-K and fig. S8), without affecting cardiomyocyte proliferation (fig. S9). Thus, depletion of *hmmr* resulted in decreased regenerative capacity of the zebrafish heart.

3.4 Inhibition of Has and Hmmr blocks epicardial EMT and their migration

Previous work demonstrated the importance of epicardial cell activation and migration into the clot to support angiogenesis (*14*). We observed a marked reduction in vessels structures in the regenerating ventricle after HMC or *hmmrVMO* injections, suggesting a defective epicardial response after injury. The lack of coronary vasculature could have arisen from a failure of either activation of epicardial cells or their subsequent EMT and migration into the clot tissue. We determined that HA was localized within the epicardium implicating a role for HA in this tissue (fig. S10A). Previous work have shown that HA can stabilize Integrin signaling through phosphorylation of Focal Adhesion Kinase (FAK) thus facilitating proper cell migration (*36*). FAK is a non-receptor cytosolic tyrosine kinase that is present at the sites of contact between cells and the ECM (*37*). In epicardial cells that have migrated into the clot pFAK (Y-397) was detected in a punctate pattern (fig. S10B). To test whether HA is required for epicardial cell activation and migration, HMC was injected into *Tg(wt1b:EGFP)* zebrafish after ventricular resection. Although epicardial Wt1b:EGFP^{+} cells activation was similar between PBS and HMC injected fish (fig. S10C, D), we observed diminished number of EGFP^{+} cells inside the clot (Fig. 5A-E) in HMC-injected hearts. HMC treatment also reduced pFAK levels in epicardial cells (fig. S10E, F, and G). Moreover, in PBS injected hearts, Wt1b:EGFP^{+} cells appeared more round in shape rather than elongated, indicating by morphology that epicardial cells are undergoing EMT (Compare Fig. 5F to G & fig. S10F). To confirm that epicardial EMT was suppressed after HMC injections, we performed Q-PCR to measure expression of EMT genes (*16*). At 3dpa, HMC injections reduced the expression levels of *snail1a*, *snail1b*, *snail2* and *twist1a* (Fig. 5H & J), supporting the notion that HA is required for epicardial EMT. These findings were confirmed with knockdown of Hmmr as presence of epicardial pFAK was reduced (Fig. 5J-L). Our studies suggest a role where HA and its receptor Hmmr are required for proper epicardial cell EMT and migration into the wound.

3.5 HA signals through FAK and Src for Epicardial EMT

We next addressed the potential role for FAK and Src in heart regeneration as downstream effectors of HA/Hmmr. FAK is autoactivated through tyrosine autophosphorylation at the Y397 site by Integrin/Hmmr interaction and is known to be a critical regulator of cardiac growth and remodeling (*38*). This pY397 creates a high-affinity binding site for the SH2 domain of Src family kinases that leads to further phosphorylation of FAK by Src (*39*). The activation of

FAK/Src complex then regulates downstream signaling pathways that control cell proliferation, spreading, motility and EMT (*40*). In this study we used chemical inhibition of FAK with PF-573228 (*41*) and the Src kinase inhibitor (SKI-1) to suppress Src (*42*), to dissect the role of these proteins in heart regeneration. Inhibiting either FAK or Src, suppressed heart regeneration (Fig 6 A-D and fig S11 A,B). As observed with blocking HA production, the formation of new vessels was also significantly reduced after injection of PF-573228 or SKI-1 (Fig 6 E-N & fig. S11 C-F). and the migration of epicardial cells inside the clot was reduced after SKI-1 injection (fig. S11 G,H), suggesting that Hmmr activation of pFAK and Src are important for epicardial cell function.

In support of these findings we employed an *ex vivo* assay to monitor epicardial cell migration from intact hearts grown in culture (*26*). Decreased epicardial cell spreading from the heart on fibrin coated wells after *hmmrVMO* injections was observed (Fig. 7A-B, and F), Moreover, suppressing FAK (Fig. 7C-D and G), HA production (Fig. 7E) or Src activity (Fig. 7H) also reduced epicardial cell spreading. Taken together, our results implicate a function for HA and Hmmr in directing activated epicardial migration through FAK and Src kinases.

3.6 Accumulation of HA and HMMR is conserved in a mammalian model of MI

In contrast to zebrafish, after MI, adult mammals fail to regenerate injured myocardial tissue. One key difference between these animals is that in mammals cardiomyocytes are post mitotic, often existing as binuclear or polyploid cells (*43*). However there are some similarities with respect to how the epicardium responds to injury (*44*). HA has been reported to accumulate after injury in many tissues and in different species (*28, 45*), but the presence and function of HMMR remains understudied in injured mammalian hearts. In uninjured rats hearts neither HMMR, nor HA, were detected (fig. S12A, D, G). However, after MI, HMMR and HA were detected in the scar tissue at 7 days post MI (dpMI) (fig. S12B, E, H) and at 8 weeks post MI (wpMI), but not in the myocardium (fig. S12C, F, I). These data support the notion that there is an evolutionary conserved response to cardiac damage with the production of HA and induction of its receptor in scar tissue.

4. Discussion

Using a proteomic approach we identified increased expression of Atp5a1, Desmuslin, and Hmmr at 3 dpa. Atp5a1 is a critical enzyme in the energetic pathways of cells, and its induction can be interpreted as increasing of energy demand of the damaged heart. This is consistent with results in rat ischemic myocardium where ATP synthase expression was increased (*46*). *Desmuslin* is an intermediate filament, important for the structure of the cell. During embryogenesis, mutation of *Desmuslin* correlates with the development of cardiomyopathies (*47*). *Desmuslin* could play a role in the activation and replacement of contractile apparatus, maintaining the cell integrity and restoring the damaged cells in the injured area.

Hmmr is a receptor for HA, and its main function is to promote cell motility in wound healing. Previous studies indicate that HA, *has2* and *cd44* are important for mesenchymal cell proliferation during tail regeneration in *Xenopus laevis* tadpoles (*28*). During development Has2 plays an essential role in endocardial cushion, cardiac valve and epicardium formation (*48-50*) *Has2* knockdown mice die during midgestion (E9.5-E10), and have severe cardiac and vascular deformations and reduced body size (*48*). In zebrafish, *has2* is strongly expressed in cardiac progenitor cells and is necessary for cardiomyocyte migration and cardiac cone rotation (*27, 51,*

52). Here, we report that HA accumulates in regenerating zebrafish hearts that correlates with cardiac injury studies performed in newt, where ECM components tenascin-C, HA, and fibronectin were up-regulated (*53*). In zebrafish, fibronectin accumulates in the wound following injury and promotes the integration of regenerating cardiomyocytes (*54*), suggesting that ECM components provide physical bioscaffold to promote regeneration. HA, a non-sulphated glycosaminoglycan, is often described as an ECM component, but HA has also been demonstrated to be required for cell proliferation and migration (*55*). Moreover, its function in the developing embryo in heart formation suggests that HA is not just a passive component of the ECM (*56*). In this study we focused on the potential role of HA and its receptor Hmmr in regulating cellular migration and proliferation during cardiac regeneration. We observed that decreased production of HA reduced epicardial cell EMT and their migration into the injured area, which is an important step to support coronary vasculature formation. This is supported by the observation that Wt1bEGFP^{+} cells appeared morphologically to be elongated after suppressing Hmmr and by blocking HA synthesis. In addition, the phosphorylation of FAK in Wt1EGFP^{+} cells was decreased as well as diminished expression of *snail* and *twist* genes suggesting that HA is important for epicardial cell EMT. Furthermore, chemical inhibition of FAK activity also resulted in diminished epicardial cell migration, supporting previous studies that show that disruption of HA-HMMR interaction reduced cell motility and formation of stable focal adhesion (*57*). Given that specific FAK tyrosine phosphorylation at position 397 is known to activate Src (*36*), we also observed a similar lack of epicardial cell migration into the wound in zebrafish treated with a Src Kinase Inhibitor. Taken together, these studies suggest that HA and its receptor *hmmr* play a critical role in epicardial cell EMT after injury and involves FAK and Src signaling. Our data implies that HA is not only a component of the ECM, but could function as an instructive molecule that promotes epicardial EMT. Future studies using tissue and cell specific gene knockout of components in this pathway will determine their exact function in epicardial cell EMT and zebrafish heart regeneration.

The relevance of these findings to mammals is supported by the observation that expression of HMMR and HA was induces in rat hearts following ischemic injury. Other studies have shown that application of HA based hydrogels into rat hearts after MI can induce neovascularization, and more important, improve cardiac function (*58*). These findings open the possibility of increasing HA in the mammals to improve cardiac repair after ischemic injury. Our study highlights the importance of HA and *hmmr* and provide some mechanistic insights into the role of HA in epicardial EMT to promote neovascularization in the regenerating zebrafish heart.

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Conflict of Interest
None declared.

Figures Legends

Figure 1. Increased expression of *hmmr*, *cd44*, *has-1* and *-2* and HA after ventricular resection. (**A**) ISH in hearts showing that *hmmr* is highly expressed at 3 dpa (n=5), compared to the uninjured hearts (n=5). *myl7* labels myocardium. A= Atrium; V= Ventricle; BA= *Bulbus Arteriosus*. Black dashed lines delimitate the injured area. (**B**) Q-PCR analyses of *hmmr*, *cd44*, *hyaluronan synthase 1* (*has 1*), *2*, and *3*, and *hyaluronidases 2* (*hyal2*) expression at 1, 3, and 7 dpa. Values were normalized to *α-actin* and *RNAP* expression. (**C**) AFOG staining of uninjured hearts (n=6) and hearts at 3 (n=13), 7 (n=11), 14 (n=5) and 30 dpa (n=5). AFOG stains intact cardiac muscle in yellow-orange, fibrin in red, and collagen in blue. Confocal images of *Tg(myl7:EGFP)* hearts showing intact cardiac muscle in green and accumulation of HA after injury in red. HA, detected using biotinylated HABP (bHABP), was found in proximity of the clot (arrows). White boxes show the area where the higher magnification picture was taken. Sections were counterstained with DAPI (blue). Scale bars, 100 μm.

Figure 2. Suppressing HA production blocked cardiac regeneration. (**A**) Experimental outline. Amputation of ventricle apex was performed at day 0. At 1 dpa zebrafish were retro-orbital injected daily to deliver HMC or PBS. (**B-E**) HMC injections decreased HA levels in the wound. AFOG staining of *Tg(myl7:EGFP)* hearts at 3 dpa injected with PBS (n=7) (**B**) or with HMC (n=7) (**C**). Black boxes outline area of higher magnification images. Black arrowheads mark scar tissue. (**D**) HA (red) (white arrowheads) was detected within the injury site in control PBS injected, but was markedly diminished after HMC injection (**E**). (**F, G**) Hearts at 30 dpa had minimal or no scar tissue in PBS controls (n=26) (**F**), but after HMC injections (n=21) significant scar tissue was observed (**G**). Black arrowheads mark scar tissue. (**H**) Graph of measured scar tissue at 30 dpa in PBS and HMC injected zebrafish. Black bars indicate the mean and error bars the SEM. (**J, I**) αSMA staining for myofibroblasts in *Tg(myl7:EGFP)* hearts at 15 dpa, injected for 6 days with PBS (**I**; n=4) or HMC (**J**; n=5). Sections were counterstained with DAPI (blue). White arrows indicate αSMA staining. Scale bars, 100 μm. *** P<0.001. Student's *t*-test.

Figure 3. Has inhibition suppressed angiogenesis in regenerating hearts. (**A**) *Tg(fli1a:EGFP)* hearts at 10 dpa, injected for 6 days with PBS (n=9) and (**B**) HMC (n=5). Mef2c immunostaining revealed presence of cardiomyocytes (white arrowheads) that have populated into the clot area in PBS controls, (**A**), but was clearly decreased after HMC treatment (**B**). White dashed lines delineate the resection plane. The formation of coronary vasculature at 10 dpa was observed through endothelial EGFP expression (red arrows) in *Tg(fli1a:EGFP)* hearts (**C**). In HMC treated zebrafish (**D**), the presence of $EGFP^+$ cells was reduced. (**E-F**) Merged images of **A** & **C** and **B** & **D**. DAPI is shown in blue. (**G**) Graph showing cardiomyocyte number in the regenerating area. (**H**) Quantification of angiogenesis after injection of HMC and PBS, expressed as new vessel area inside the clot. (**I-J**) Hearts at 10 dpa, injected for 6 days with PBS (**I**; n=5) or HMC (**J**; n=5) stained for Fli1b expression and MHC. (**K**) Quantification of $Fli1b^+$ cells per unit clot area. Black bars in graphs indicate the mean and error bars the SEM. Scale bars, 100 μm. *P<0.05; **P<0.01. Student's *t*-test.

Figure 4. Cardiac regeneration is inhibited after Hmmr knockdown. (**A-C**) AFOG staining of hearts at 30 dpa injected for 10 days with PBS (n=12) (**A**), 5 bp mismatch *VMO* (*hmmr-MutMO*) (n=13) (**B**), and *hmmrVMO* (n=12) (**C**). Red boxes indicate the scar tissue area. Zebrafish injected with *hmmrVMO* contained extensive scar tissue at 30 dpa. (**D**) Graph depicting clot area at 30 dpa. (**E-J**) Hearts at 10 dpa showing clot by AFOG staining (**E-G**), and cardiomyocytes (Mef2c) and coronary vasculature (Fli1a:EGFP^{+}) (**H-J**). Injection of *hmmrVMO* resulted in decreased Fli1a:EGFP^{+} cells into the injury site (**J**; n=8), compared to controls (**H**; n=16 & **I**; n=6). (**K**) Quantification of Fli1a:EGFP^{+} cells in the clot area at 10 dpa after Hmmr knockdown. Black boxes indicate the area where the confocal picture was taken. White dashed lines delimitate the injured area. DAPI is shown in blue. Black bars in graphs indicate the mean and error bars the SEM. Scale bars, 100 μm. *P<0.05; **P<0.01; ***P<0.001 ns, not significant. One-way ANOVA.

Figure 5. HA is required for epicardial cell EMT. (**A-D**) *Tg(wt1b:EGFP)* zebrafish hearts at 5 dpa injected with PBS (n=8) (**A-C**), or HMC (n=6) (**B-D**) had similar clot area, as shown by AFOG staining (**A, B**), but the number of Wt1b:EGFP^{+} cells in the clot was greatly reduced after HMC injections (**D**), compared to PBS (**C**). Black boxes demarcate area of higher magnification. (**E**) Graph showing the number of Wt1b:EGFP^{+} cells in the clot at 7 dpa. (**F, G**) Confocal images of activated wt1b^{+} cells at the epicardial region at 3 dpa after injection of control PBS (**F**; n=4) or HMC (**G**; n=5). In controls (**F**), some Wt1b:EGFP^{+} cells were pFAK positive (white arrowhead) and appeared round in shape. In contrast, Wt1b:EGFP^{+} cells after HMC injections (**G**) remained elongated. (**H**) Q-PCR analysis of *snail1a*, *-1b*, *-2* and *twist1a* expression in ventricular tissue at 3dpa after injections of HMC. HMC reduced the expression of EMT genes. N indicates the number of independent replicates performed for each gene. (**I**) Western blot analysis of Twist1 expression in uninjured hearts and hearts at 1 dpa. Increased Twist1 expression was detected in resected hearts, but was suppressed after HMC injections. Total Erk2 was used as loading control. (**J-L**) *Tg(wt1b:EGFP)* heart at 3 dpa immunostained for pFAK after injection of PBS (**J**; n=4), control *mutant MO* (**K**; n=4) or *hmmrVMO* (**L**; n=3). *hmmrVMO* decreased the number of round Wt1b:EGFP^{+} cells and the number of cells expressing pFAK (**L**). Black bars in graphs indicate the mean and error bars the SEM. Scale bars, 100 μm (**A**, **B**), 25 μm (**C**, **D**, **F**, **G**, **J**, **K**, & **L**). *P<0.05; ***P<0.001. Student's *t*-test.

Figure 6. FAK and Src inhibition suppressed angiogenesis in regenerating hearts. (**A-C**) At 30 dpa, hearts injected for 10 days with PF-573228, a FAK inhibitor (**B**) (n=10), or with SKI-1 (**C**) (n=13), a Src inhibitor, failed to properly regenerate the heart as compared to DMSO controls (**A**) (n=31). Red arrows delineate scar. (**D**) Graph showing clot area at 30 dpa after injections of PF-573228 or SKI-1. (**E-M**) PF-573228 and SKI-1 injections inhibited angiogenesis. (**E-G**) AFOG staining of hearts at 10 dpa, injected with DMSO (**E**) (n=15), PF-573228 (**F**) (n=10) or SKI-1 (**J**) (n=10). (**H-M**) *Tg(fli1a:EGFP)* hearts injected with DMSO (**H, K**), PF-573228 (**I, L**) or with SKI-1 (**J, M**), immunostained for MHC to detect resection plane. White arrows indicate new vessels formed inside the clot. (**N**) Quantification *fli1a*$^{+}$*:EGFP* area in the clot at 10 dpa. FAK and Src inhibition significantly reduced new vessel formation. Black boxes delimitate the areas of confocal imaging. Black bars in graphs indicate the mean and error bars the SEM. Scale bars, 100 μm. *P<0.05; **P<0.01; ***P<0.001 One-way ANOVA.

Figure 7. HA and its receptor Hmmr are necessary for epicardial cell migration in *ex vivo* culture (**A-D**) *Ex vivo* epicardial migration assay showing decreased Wt1b:EGFP^{+} migratory behavior after *hmmrVMO* (**B)** (n=8) and PF-573228 (**D**) (n=7) treatments compared to controls (**A**) (n=14**)** and (**C**) (n=7). Images were captured at 3 days post extraction of the heart. The migration of Wt1b:EGFP^{+} epicardial cells (red arrows) was measured from day 1 to day 3. In controls PBS, DMSO, or *hmmr-MutMO* treatments, epicardial cells migrated on the fibrin coated plates. In contrast treatment with *hmmrVMO*, PF-573228 or SKI-1 significantly suppressed epicardial cell migration. (**E-H**) Graphs showing migration of Wt1b:EGFP^{+} cells under various treatments, including suppressing HA production in the hearts with HMC (**E**) (n=13), *hmmrVMO* (**F**) (n=8), PF-573228 (**G**) **(**n=7) and with SKI-1, the Src kinase inhibitor (**H)** (n=8). Black bars in graphs indicate the mean and error bars the SEM. Scale bars, 250 μm. *P<0.05; **P<0.01; ***P<0.001. 2way ANOVA.

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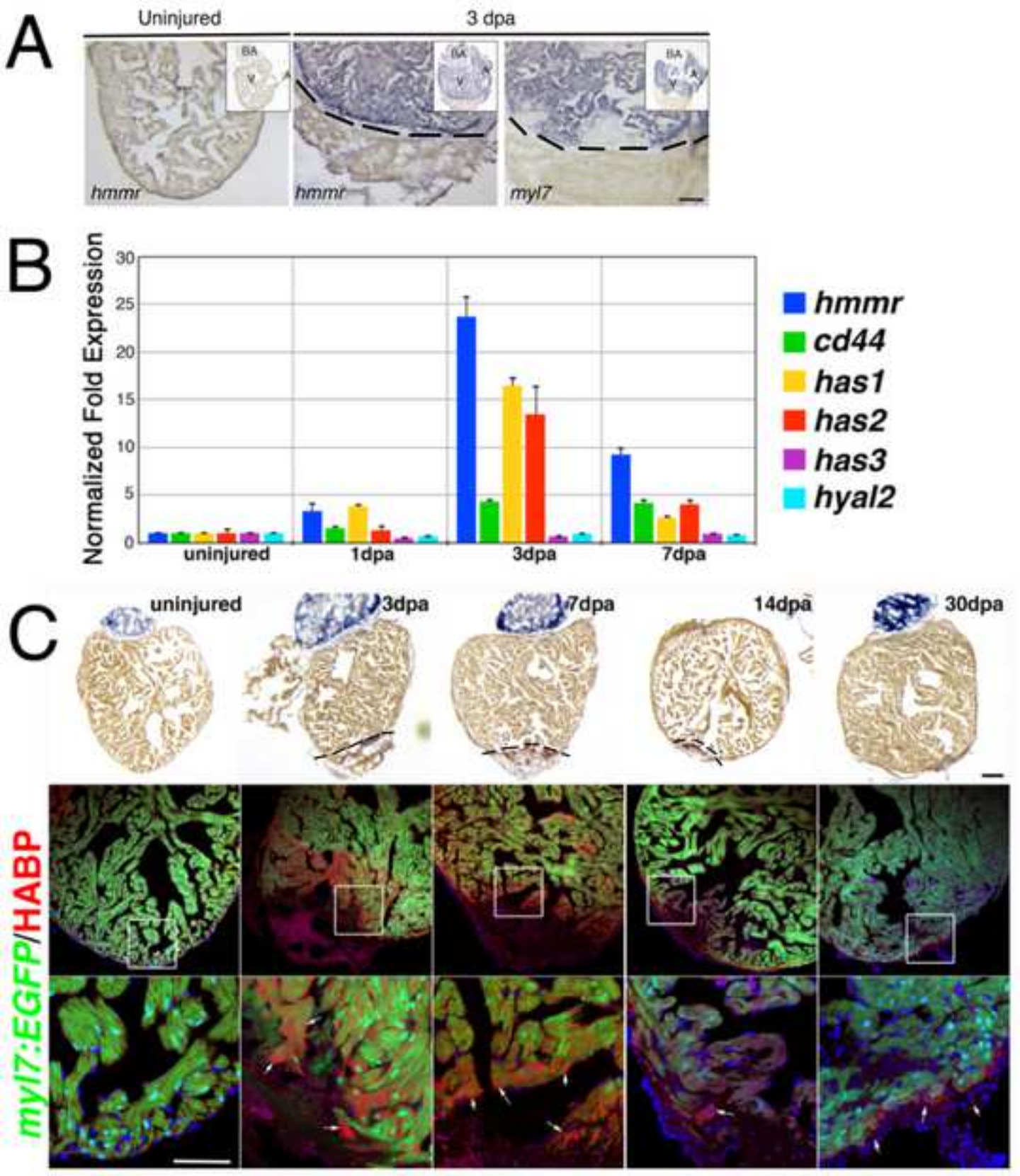

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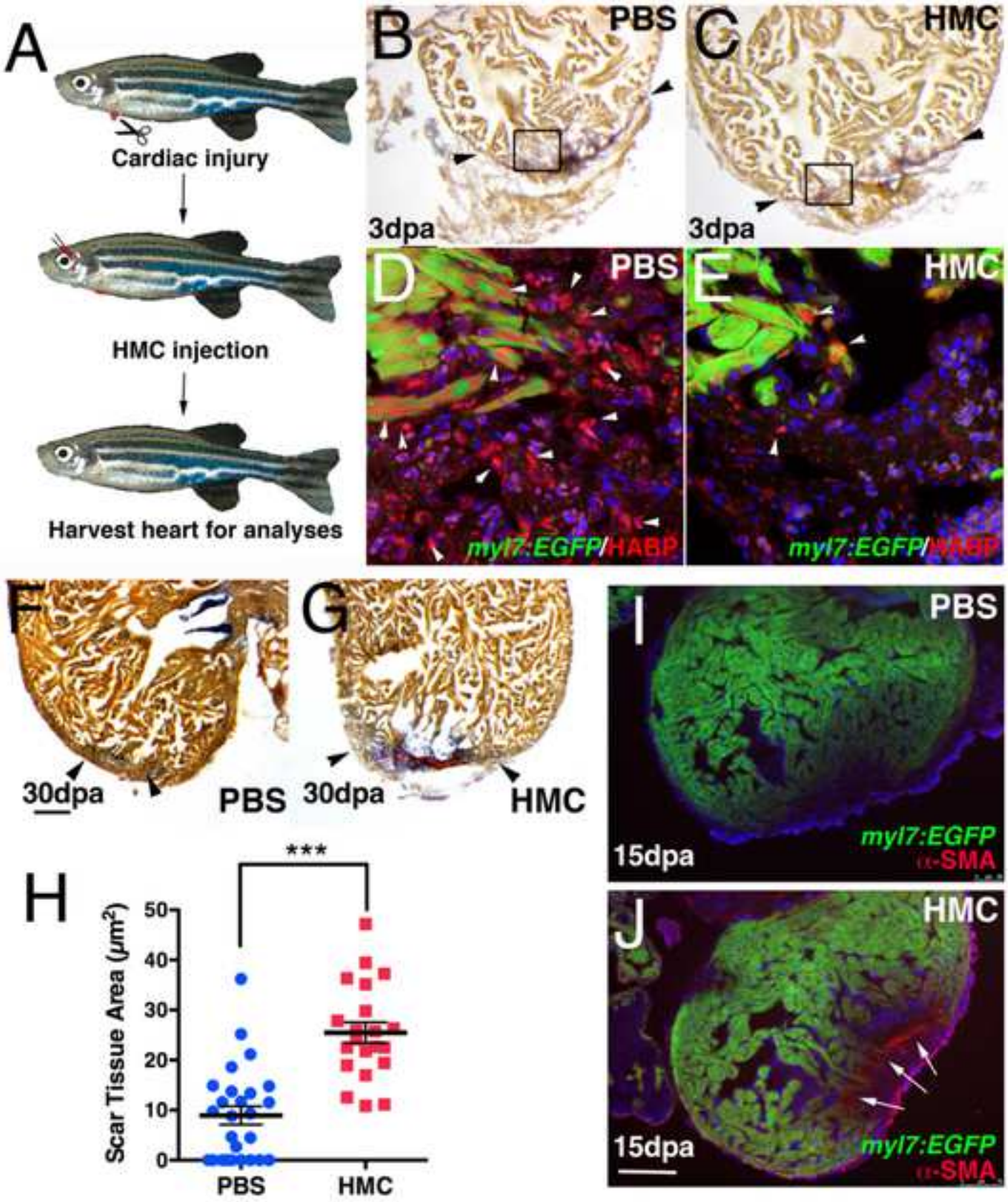

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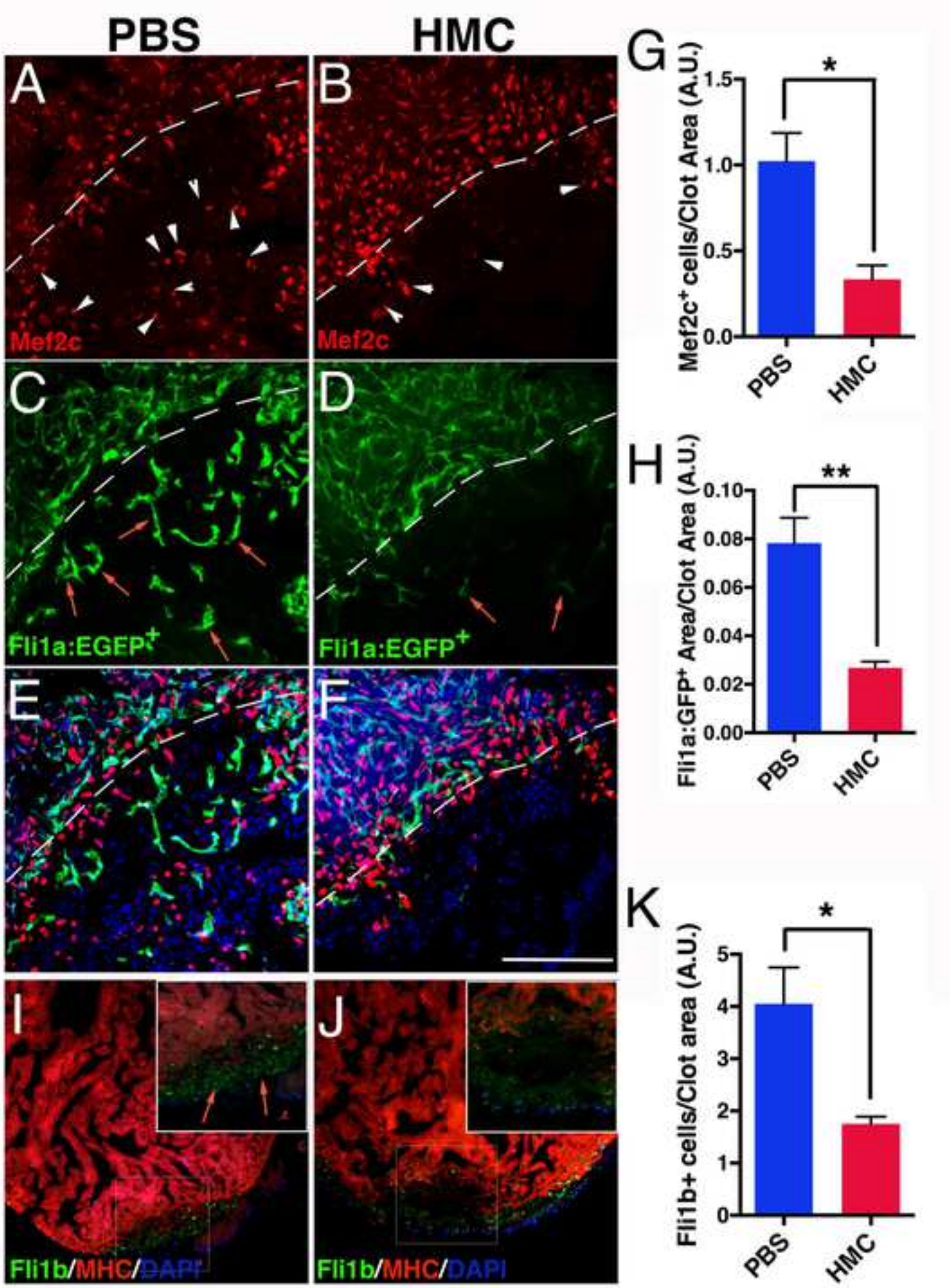

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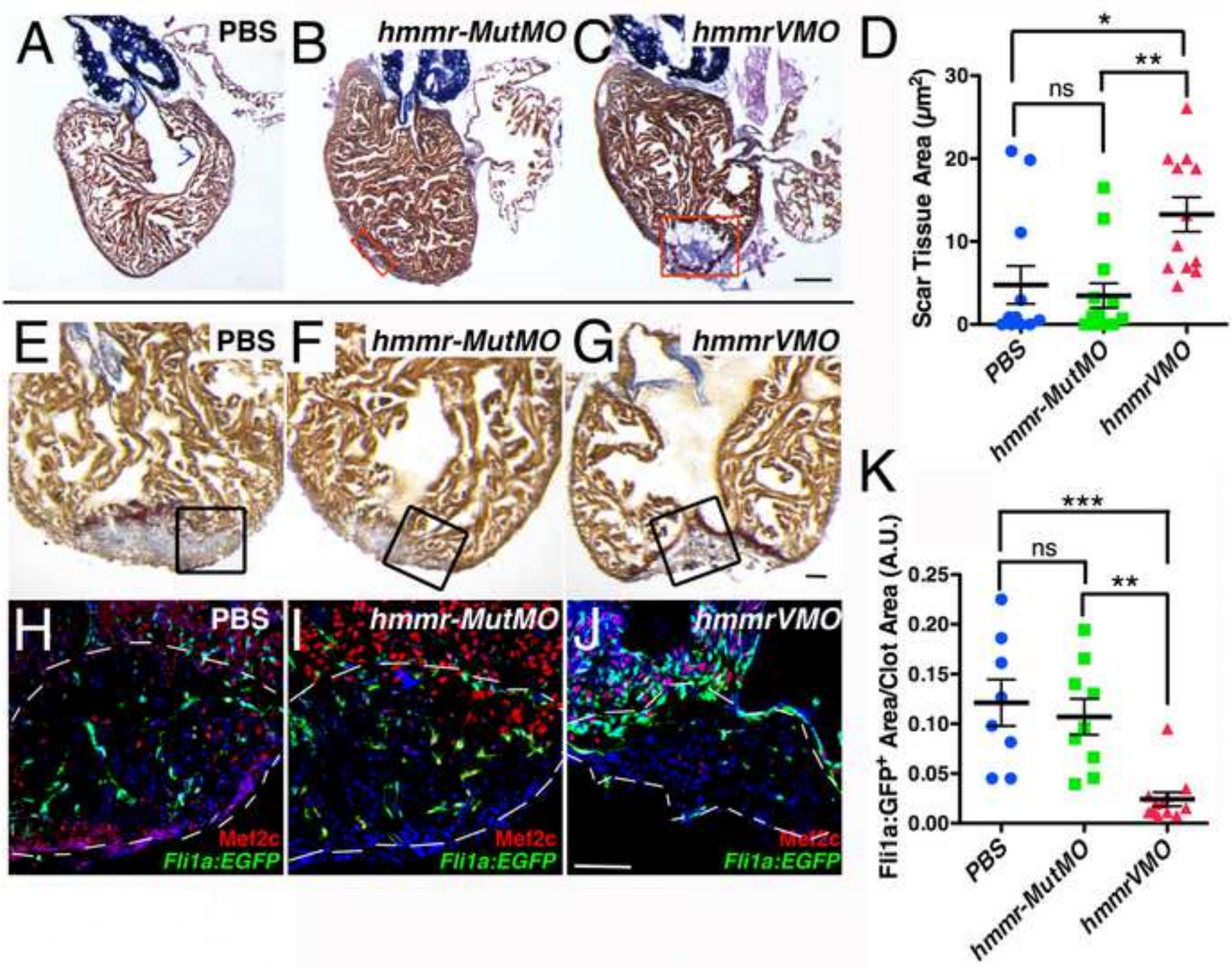

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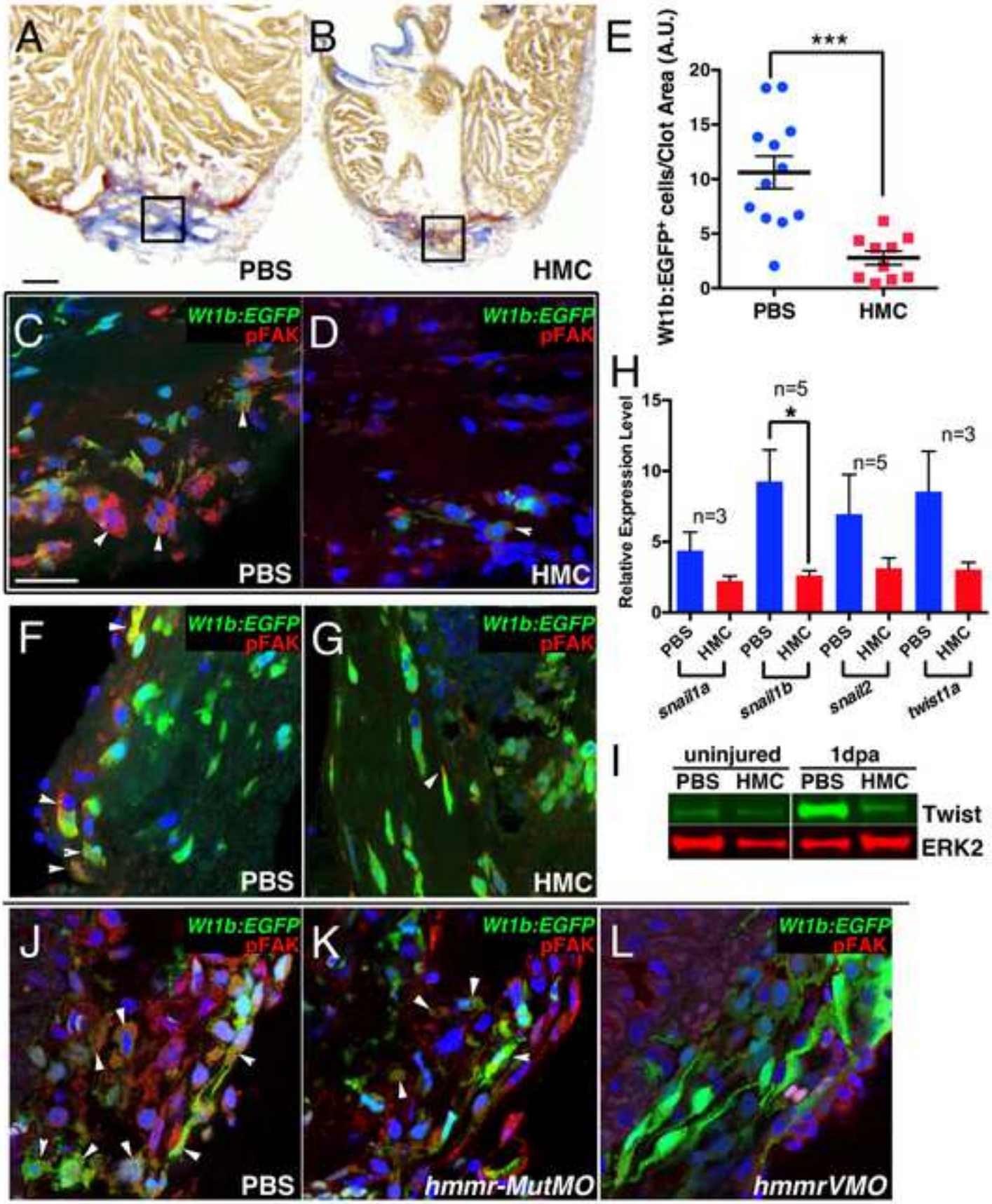

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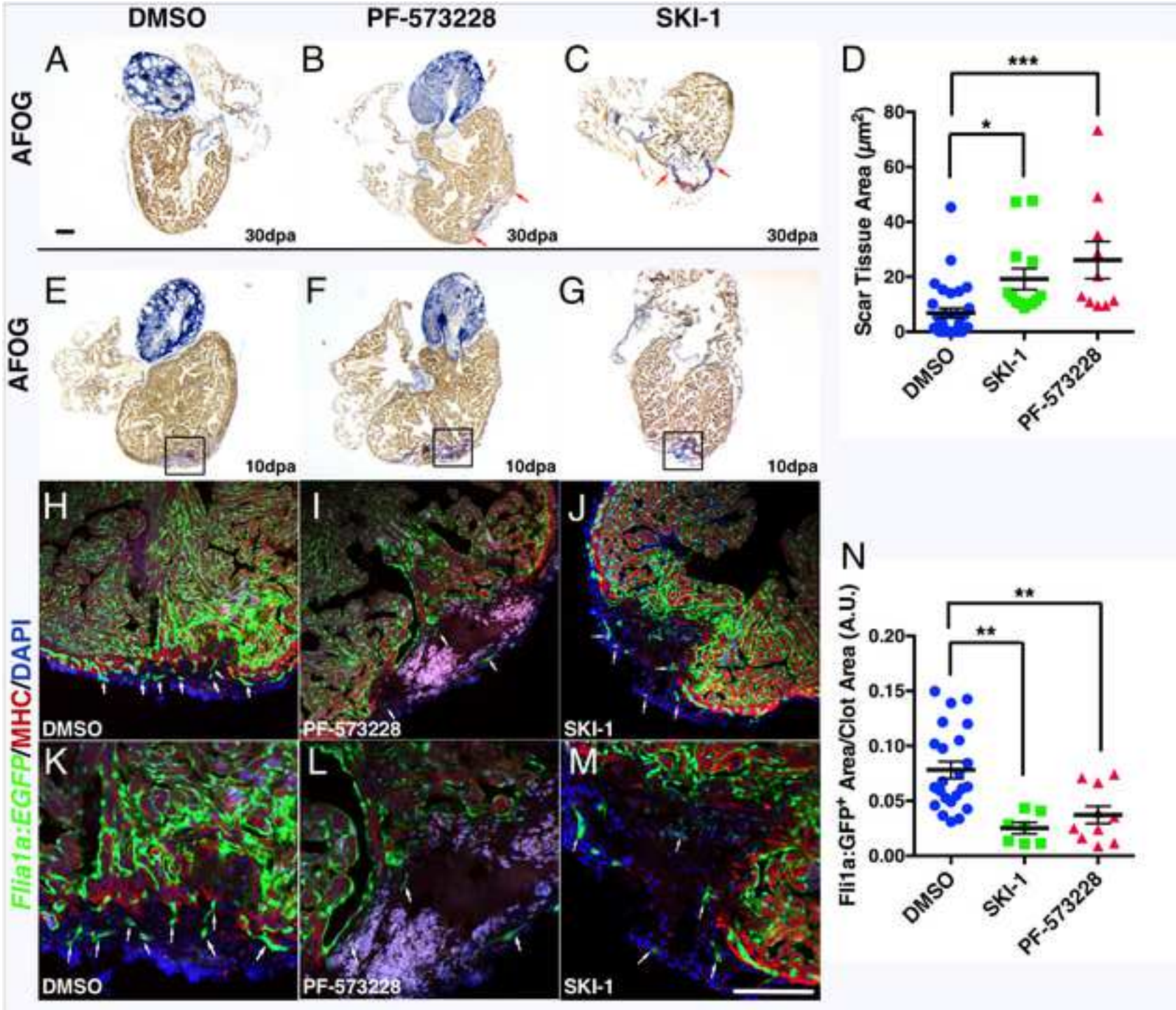

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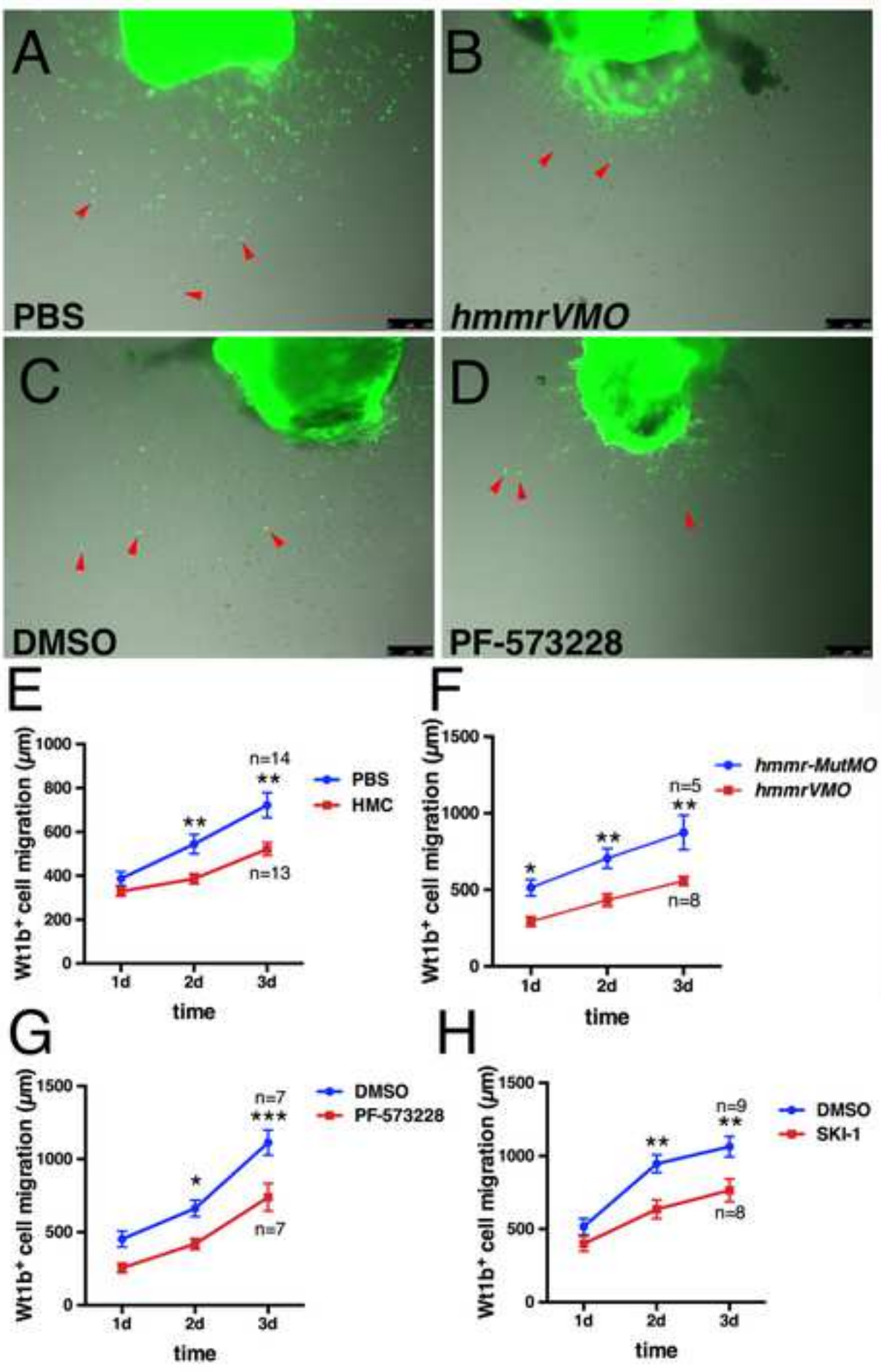

Extracellular component hyaluronic acid and its receptor Hmmr are required for Epicardial EMT during heart regeneration

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SUPPLEMENTAL MATERIAL

Methods

Clot area measurement and cell counting

Clot areas were measured using ImageJ (NIH). For each heart, the clot area values were measured as the average of the sum of the clot area of the four largest sections. Primary antibody used for immunostaining were: anti-alpha smooth muscle actin (α-SMA, GeneTex) (1:200); biotinylated Hyaluronan-Binding Protein (bHABP) (Sigma) (2 µg/ml in 10% Goat serum/PBS); anti-HAS (DG42) (kindly provided by Dr. Igor Dawid) (1:10); anti-Mef2c (Santa Cruz Biotechnology) (1:500); anti-PCNA (Sigma) (1:1000); anti-fli1b (kindly provided by Dr. Nathan Lawson) (1:800); anti-Hmmr (H-90) (Santa Cruz Biotechnology) (1:50); anti-FAK [pY397] (Invitrogen) (1:200); anti-MHC (F59) (DSHB) (1:50). Secondary antibodies (Invitrogen) used were: Alexa Fluor 488 goat anti rabbit IgG Peroxidase Conjugate (1:1000); Cy3 goat anti rabbit (1:500); Strepatvidin, Alexa Fluor 568 conjugate (1:1000), Alexa Fluo 594 goat anti mouse IgG (H+L) (1:500). Slides were mounted with Vectashield mounting medium with DAPI (Vector Laboratories). Images were taken with Zeiss Lsm 700 confocal microscope and with Leica M205 FA fluorescent stereomicroscope. For each experiment, four sections were analyzed for each heart. Slides stained with secondary antibody were used as negative control. Cardiomyocyte proliferation index was calculated as percentage of number of $Mef2c^{+}$-$PCNA^{+}$ cells divided by the number of $Mef2c^{+}$ cells. New vasculature was calculated measuring the areas of vessels formed in the clot in *fli1a:EGFP* hearts, and dividing the total clot area. Quantification of epicardial cells was done with ImageJ, measuring the areas of *wt1b:EGFP*$^{+}$ cells in the clot, and dividing for the clot area.

Table 1: Primers set for Q-PCR

Gene	Sequence 5' to 3'	Amplicon Length (bp)
RNAP F	CCAGATTCAGCCGCTTCAAG	
RNAP R	CAAACTGGGAATGAGGGCTT	149
ß-actin F	CGTGCTGTCTTCCCATCCA	
ß-actin R	TCACCAACGTAGCTGTCTTTCTG	86
atp5a1 F	CTGGTCTGAAGGGTATGTCTCTGA	
atp5a1 R	CCTGTTCTCTTGACGATGTCTCC	106

desmuslin F	CTCAGTATCAGGCTATTATTGAGGAA	
desmuslin R	GAGCGAGACCCATCTTGACC	189
hmmr F	GAATAAAGACCTTGAGGACCTACATC	
hmmr R	CTTCATTCGCTGAGTCCAAGTAAC	146
cd44 F	CAGCACTTCTGTCTGCATTG	
cd44 R	TCTTCACCTGTGTCAGATTGG	189
has1 F	GATTATGAGGTGCTGTTTGAA	
has1 R	TGAGTCACATACCTGTATGAAAT	168
has2 F	ACCCGAAGAAACTGAAGAAT	
has2 R	CTGAATCACAAACCTGTACATAG	173
has3 F	AAGAGGCTGCACGAGTGG	
has3 R	AATCACATACCTGCATGTAATCC	141
hyal2 F	CAGATCAGGTCTGAAATGAAGA	
hyal2 R	AGAGCATCAGTAATAACACTTGA	94
snai1a F	CATCAAAAGCAGCGTTCACGTTA	
snail1a R	CAGGAAAGACCGAGGCAT	155
snail1b F	GACTGACCGGCGATATGC	
snail1b R	ATCCTCCTCTCCACTGCT	193
snail2 F	AGTCCGACAGTGTTTATTTCTC	
snail2 R	GGTTGCTGGTAGTCCATAC	121
twist1a F	TTGATGCAGCATGATTCTCG	
twist1a R	ACATTCACCGTCACAACAG	191

Difference gel electrophoresis (DiGE) and MS/MS analysis

DiGE system was used to compare the protein expression levels between zebrafish hearts at 3 dpa and uninjured hearts. 8 hearts were used for each condition. Minimal protein labeling with CyDye DiGE fluor, was carried out according to manufacturer's instructions (GE Healthcare/Amersham Biosciences). Sample was subjected to isoelectric focusing (IEF) using an Ettan IPGphor (GE Healthcare/Amersham Biosciences) and 24 cm linear, pH 3–10 Immobiline DryStrips (GE Healthcare/ Amersham Biosciences). Proteins were separated on 8-16% SDS

polyacrylamide gradient gel with a constant current of 30 mA. Fluorescent images of each CyDye were acquired using a Typhoon 9400 variable mode imager (GE Healthcare/Amersham Biosciences). Protein spots of interest were manually picked and processed for MALDI-TOF analysis. MALDI-TOF was performed using the 4800 Proteomics Analyzer (ABI, Foster City, CA). Mass spectra were individually calibrated using internal trypsin peaks (842.51 and 1045.56, *m/z*) with Data Explorer software available from ABI. Proteins were identified using ProteinProspector (University of California, San Francisco; http://prospector.ucsf.edu/) set to a mass accuracy of +/- 50 ppm to compare unknown mass fingerprints to those of known proteins in the NCBI non-redundant database using a species-specific filter for *Danio rerio.* A sample containing a 1:1 ratio between proteins from uninjured hearts and 3 dpa was labeled with CyDye2 and used as internal standard. DiGE was performed in duplicate, inverting the CyDye labeling between samples, to avoid preferential binding of proteins with the CyDyes.

Western Blot

Proteins were extracted from a pool of 5 ventricles apex of each condition, using 200 µl of Laemmli Buffer (Bio-rad), containing ß-mercaptoethanol (Fisher). Samples were heated at 95ºC for 5 minutes, and 0.1 M dithiothreitol (Calbiochem) was added before loading. After SDS-PAGE, proteins were transferred in nitrocellulose membrane (Li-cor). Membrane was blocked in Odyssey Blocking Buffer (Li-cor) for 1 hour. Antibodies were diluted in Odyssey Blocking Buffer, containing 0.2% Tween 20 (National diagnostics). Blots were scanned using Li-cor Odyssey CLx Infrared imaging system, and band intensity were quantified and normalized using Image Studio software. Primary antibody used: anti-Twist1 (Sigma) (1:1000), anti-ERK-2 (Sigma) (1:500), anti-actin (Sigma) (1:5000), anti-FAK [pY397] (Invitrogen) (1:1000). Secondary antibody used for western blot were: IRDye 800 donkey anti-rabbit IgG (H+L) (Li-cor) (1:15000), and IRDye 680 goat anti mouse (H+L) (Li-cor).

Rat maintenance and Myocardial Infarct (MI) induction

The research protocol followed the National Institutes of Health guidelines for animal care and was approved by the University of Pittsburgh's IACUC. Lewis adult female rats (200-250g) were purchased from Harlan Sprague Dawley Laboratories (Indianapolis, IN). The rats underwent endotracheal intubation and were mechanically ventilated with a small animal respirator at a frequency of 70–90/min with 1 to 1.5% isoflurane with 100% oxygen gas. The rat hearts were exposed through a left thoracotomy as described by (1). Rat coronary artery was ligated with a 7-0 polypropylene suture (approximately 1 to 2mm below left atrium and right-left ventricular border). Myocardial infarction was confirmed using cardiac MRI with FLASH cine movie mode at the left ventricular (LV) short-axis papillary muscle level and aorta-LV longitudinal plane images (TE/TR = 2.6 /5.0 msec, flip angle = 15°, Bruker 7T/30 Biospec, Bruker, Germany) at 7 and 28 days post ligation.

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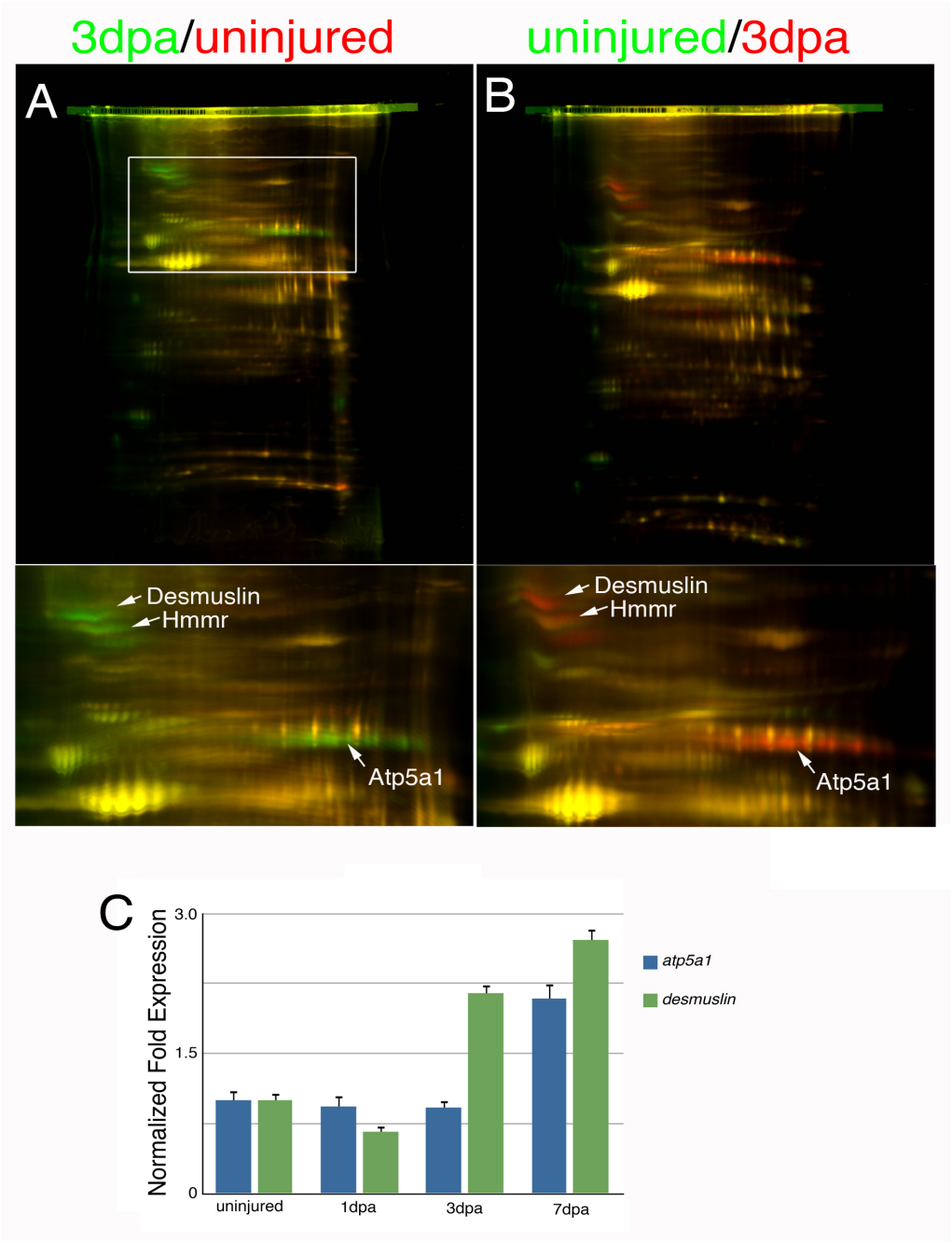

Fig. S1. A proteomic approach to discover new cardiac regeneration markers: Atp5a1, Desmuslin, and Hmmr protein levels are increased after ventricular amputation. (**S1A**) DiGE of proteins differentially expressed in adult zebrafish hearts at 3 dpa (labeled green, Cy5), compared to uninjured hearts (labeled red, Cy3). In yellow (Cy2), proteins equally expressed in the two samples. Proteins were separated in the horizontal direction by isoelectro focusing point (pI) from pH 3 to 10, and vertically by molecular weight (MW) from ~150 to 6 kDa. Magnification of the white box is shown below each gel. (**S1B**) DiGE of proteins differentially expressed in adult zebrafish hearts at 3 dpa (labeled red, Cy3), compared to uninjured hearts (labeled green, Cy5). Samples labeling was inverted to avoid preferential binding of proteins with the CyDyes to control the specificity of the reaction. (**S1C**) Q-PCR for *atp5a1* and *desmuslin* at 1, 3, and 7 dpa. Values were normalized according to *α-actin* and *RNAP* expressions. Uninjured hearts were used as control. Shown is a representative experiment from three independent replicates.

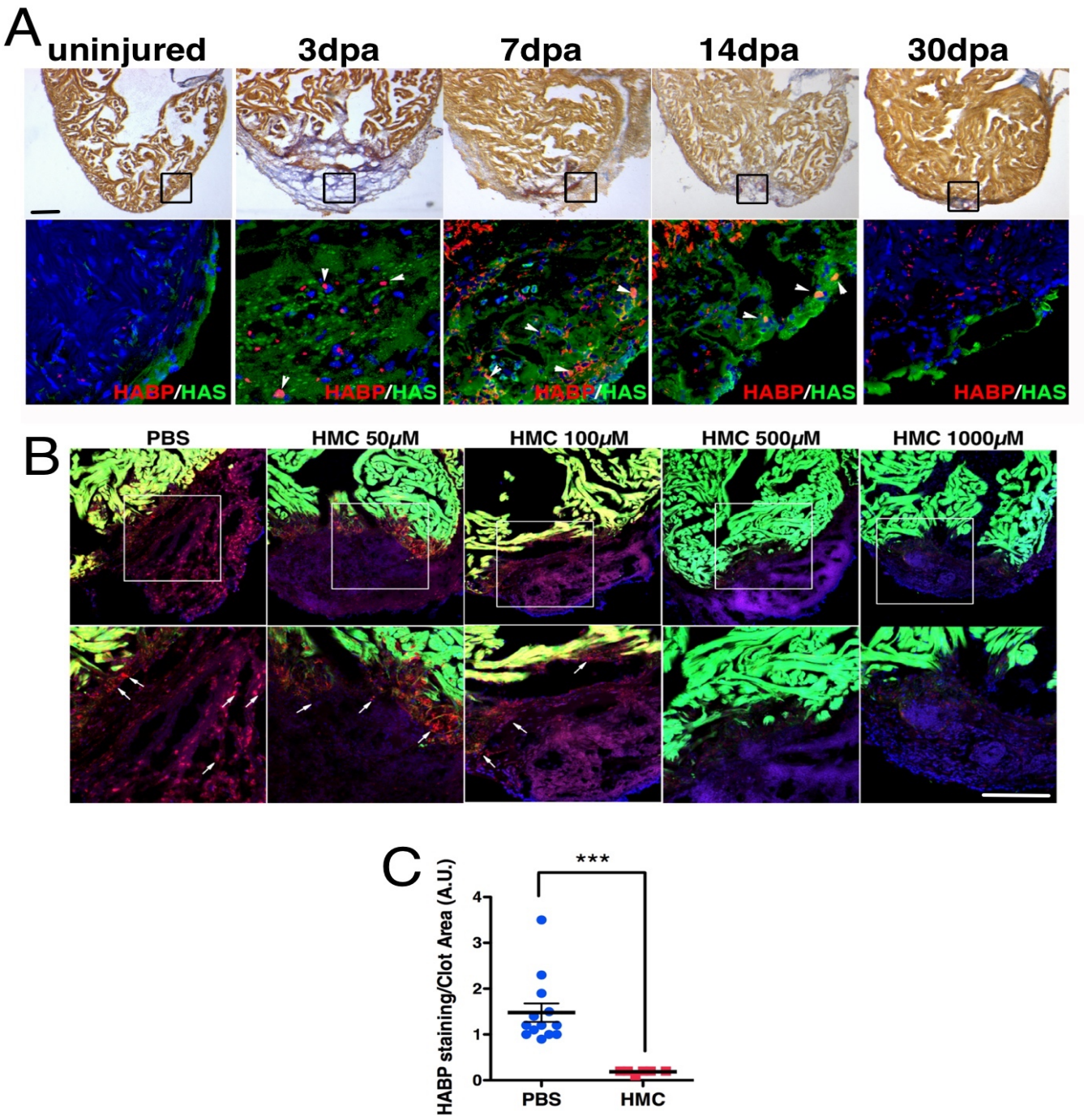

Fig. S2. Increased HA levels and Has expression after amputation.
(**S2A**) Representative images of hearts showing co-localization of HABP and Has (arrowheads) in hearts at various time point after ventricle apex amputation. Black boxes in the AFOG stained pictures show the areas where confocal pictures where taken. (**S2B**) Dose response of HMC after retro-orbital injections. *Tg(myl7:EGFP)* hearts at 3 dpa injected with increasing concentrations of HMC (n=4 for each condition). Sections are stained for HA (red) to show the efficiency of HMC in inhibiting HA synthesis in the injured area. 500 μM was used in all the following experiments. White boxes indicate the area of higher magnification. DAPI is shown in blue. (**S2C**) Quantification of HA levels in hearts after HMC injections (n=6) as compared to control PBS (n=13). Black bars in graphs indicate the mean and error bars the SEM. Student's *t*-test ***P<0.001. Scale bars, 100 μm

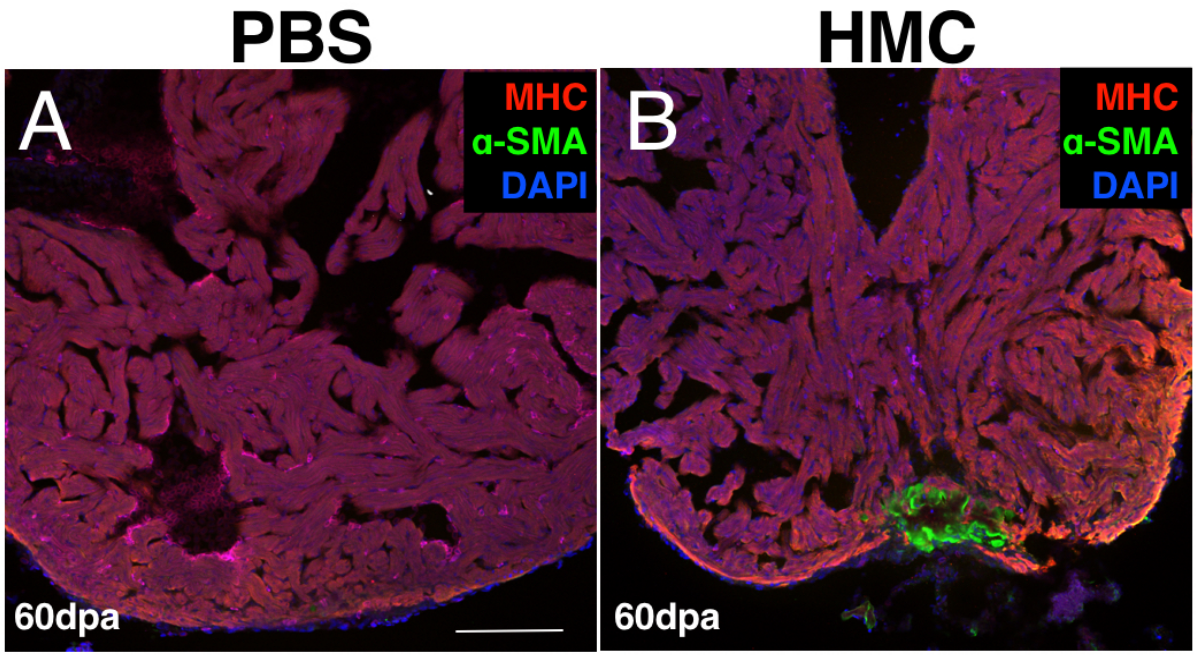

Fig. S3. HA is necessary for cardiac regeneration. (**S3A-B**) Hearts at 60 dpa, injected for 10 days with PBS (**S3A**; n=4) or HMC (**S3B**; n=2). Sections are stained with αSMA to label myofibroblasts, MHC to delineate myocytes, and DAPI for nuclei. HMC injected hearts show myofibroblasts accumulation in the injured area and fail in regenerating. Scale bars, 100 μm.

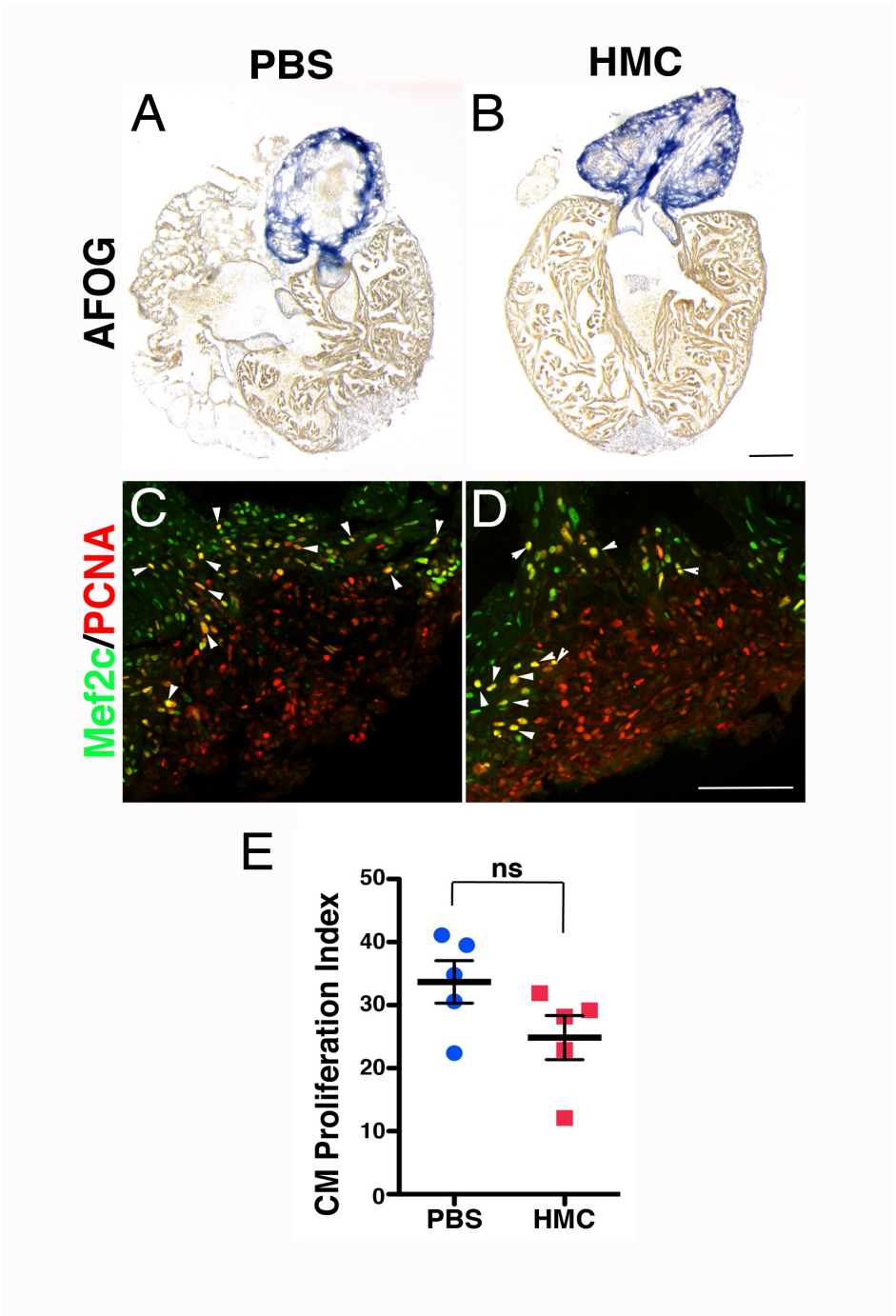

Fig. S4. Cardiomyocyte proliferation is not affected by Has inhibition. (**S4A-S4B**) AFOG staining of hearts at 7 dpa, injected for 6 days with PBS (n=6) (**S4A**) and HMC (n=7) (**S4B**) show similar clot area. (**S4C-S4D**) PCNA (red) and Mef2c (green) immunostaining showed similar cardiomyocyte proliferation index in hearts injected with PBS (**S4C**) or HMC (**S4D**). Arrowheads indicate double positive cells. Cells were counted using ImageJ software from four largest sections for each heart. (**S4E)** Graph showing the cardiomyocyte proliferation index in PBS and HMC injected hearts. Black bars in graphs indicate the mean and error bars the SEM. Data were not statistically different between the two treatments by Student's *t*-test. Scale bars, 100 μm. ns, not significant.

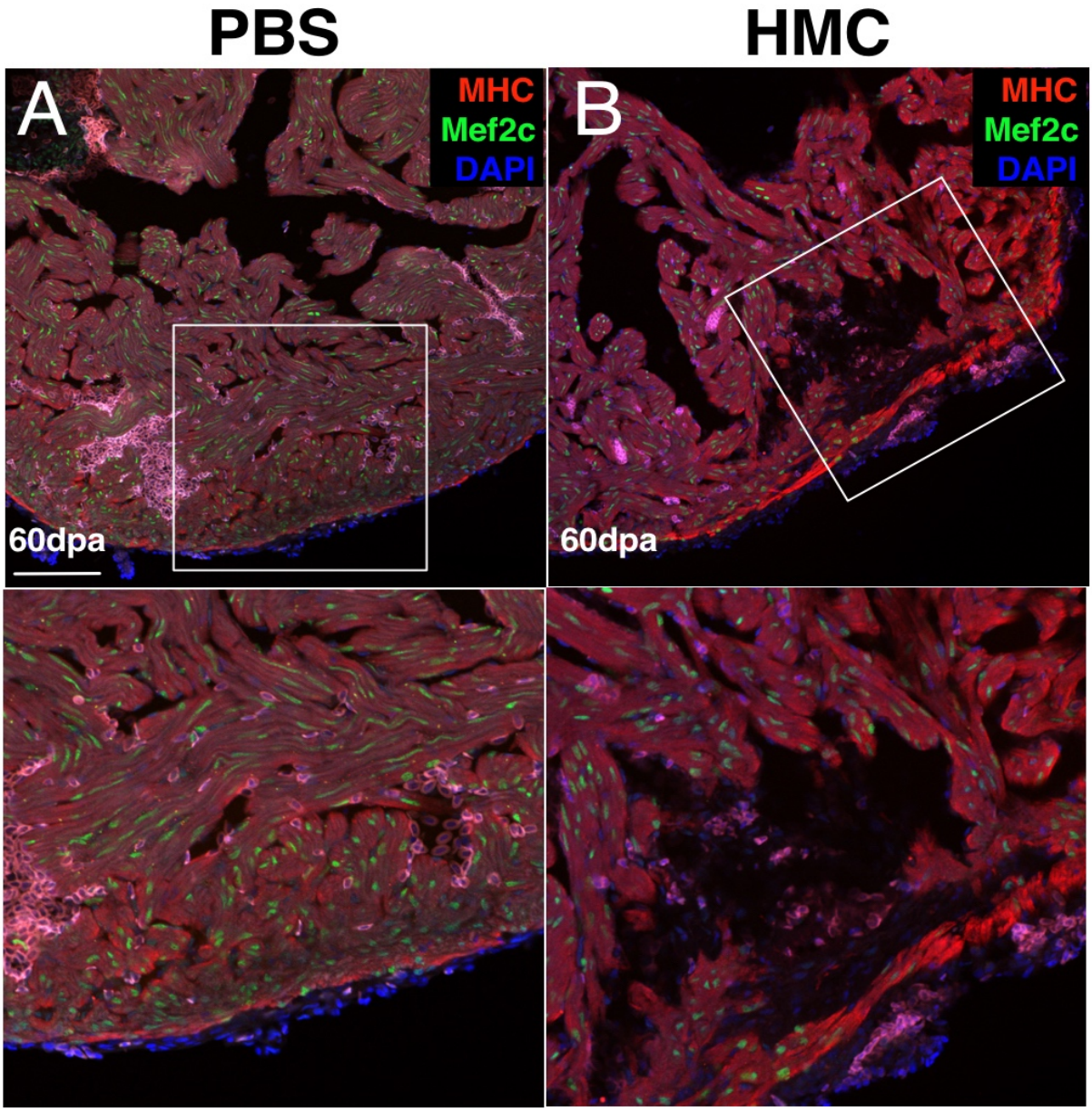

Fig. S5. HMC injection inhibit cardiac regeneration. Hearts at 60 dpa injected with PBS (n=4) (**S5A**) or HMC (n=3) (**S5B**). Sections were stained with MHC (red) and Mef2C (green) to label the myocytes and their nuclei, respectively. DAPI (blue) was used to label all cell nuclei. Zebrafish injected with HMC failed to regenerate and were devoid of cardiomyocytes inside the scar tissue area. White box show the area of higher magnification.

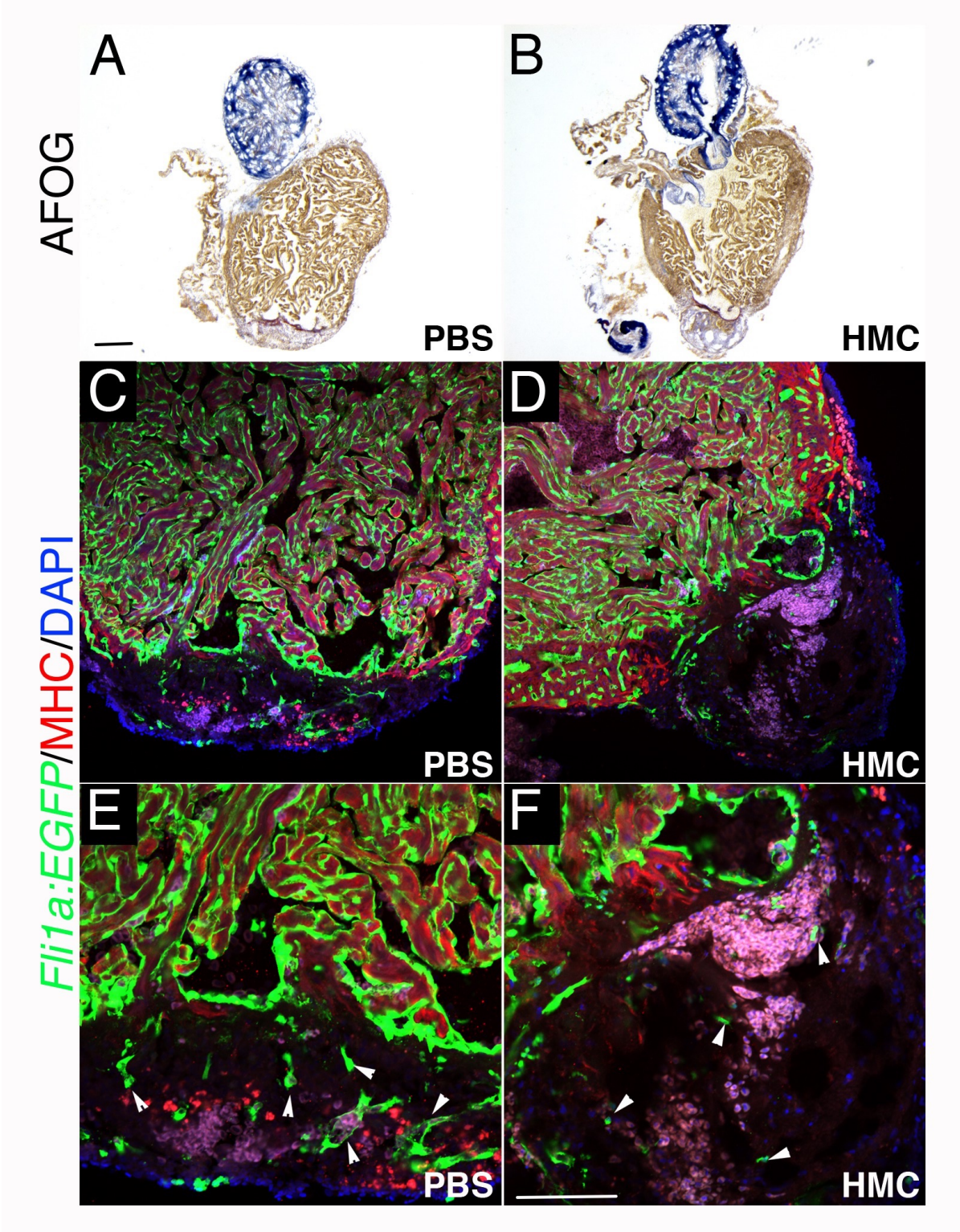

Fig. S6. HA is required for proper coronary vasculature formation. (**S6A, S6B**) AFOG staining of *Tg(fli1a:EGFP)* hearts at 10 dpa, injected for 6 days with control PBS (**S6A**; n=5), or HMC (S6B; n=5). (**S6C-S6F**) Merged confocal images showing endothelial cells (green), myocardium (red) and nuclei (blue) of control PBS (**S6C, S6E**) and HMC injected zebrafish hearts (**S6D, S6F**). The formation of new vessels in hearts from HMC injected zebrafish was reduced.

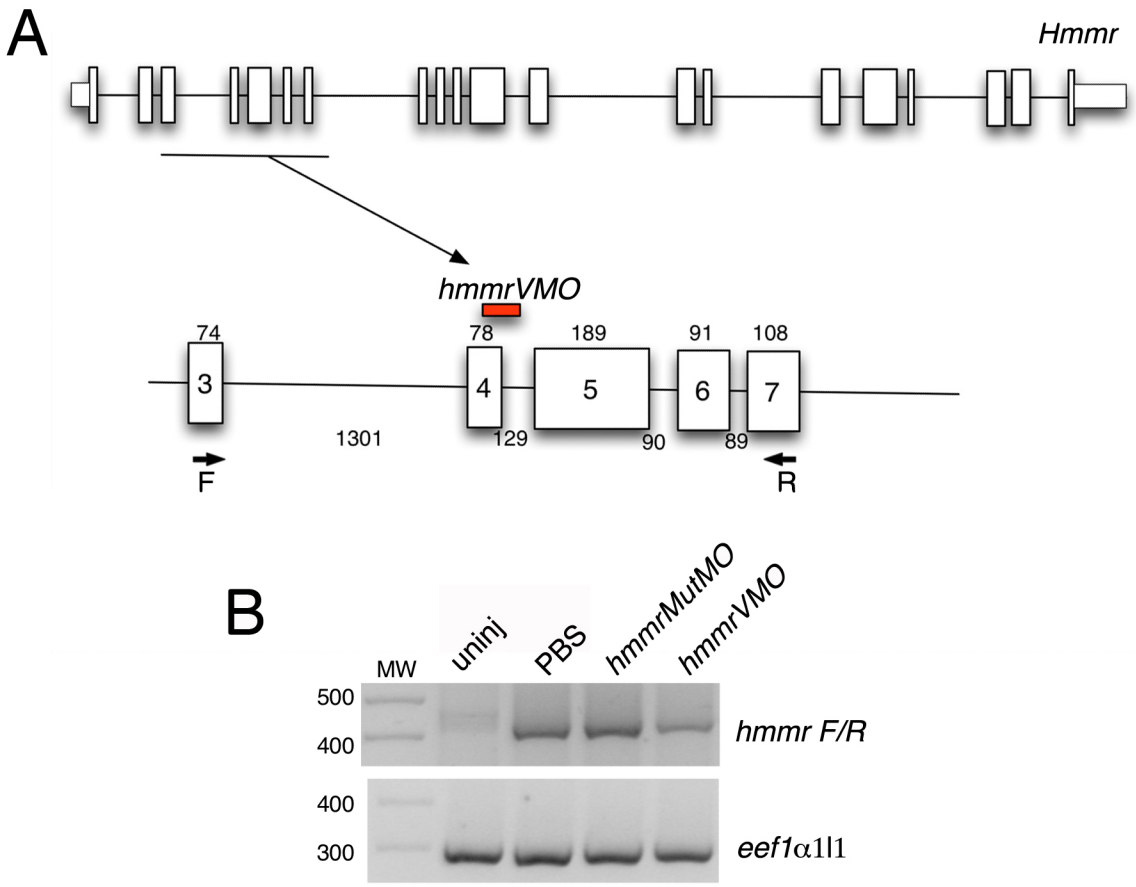

Fig. S7. Injection of *hmmrVMO* in adult zebrafish decreased *hmmr* transcripts. (**S7A**) Schematic of *hmmr* gene locus showing *VMO* targeting site. (**S7B**) PCR for *hmmr* in uninjured (uninj) hearts and hearts at 3 dpa injected with PBS, 5 bp mismatch *VMO* (*hmmrMutMO*), or *hmmrVMO*. *Eukaryotic translation elongation factor 1alpha 1, like 1 (eef1a1l1)* was used as positive control.

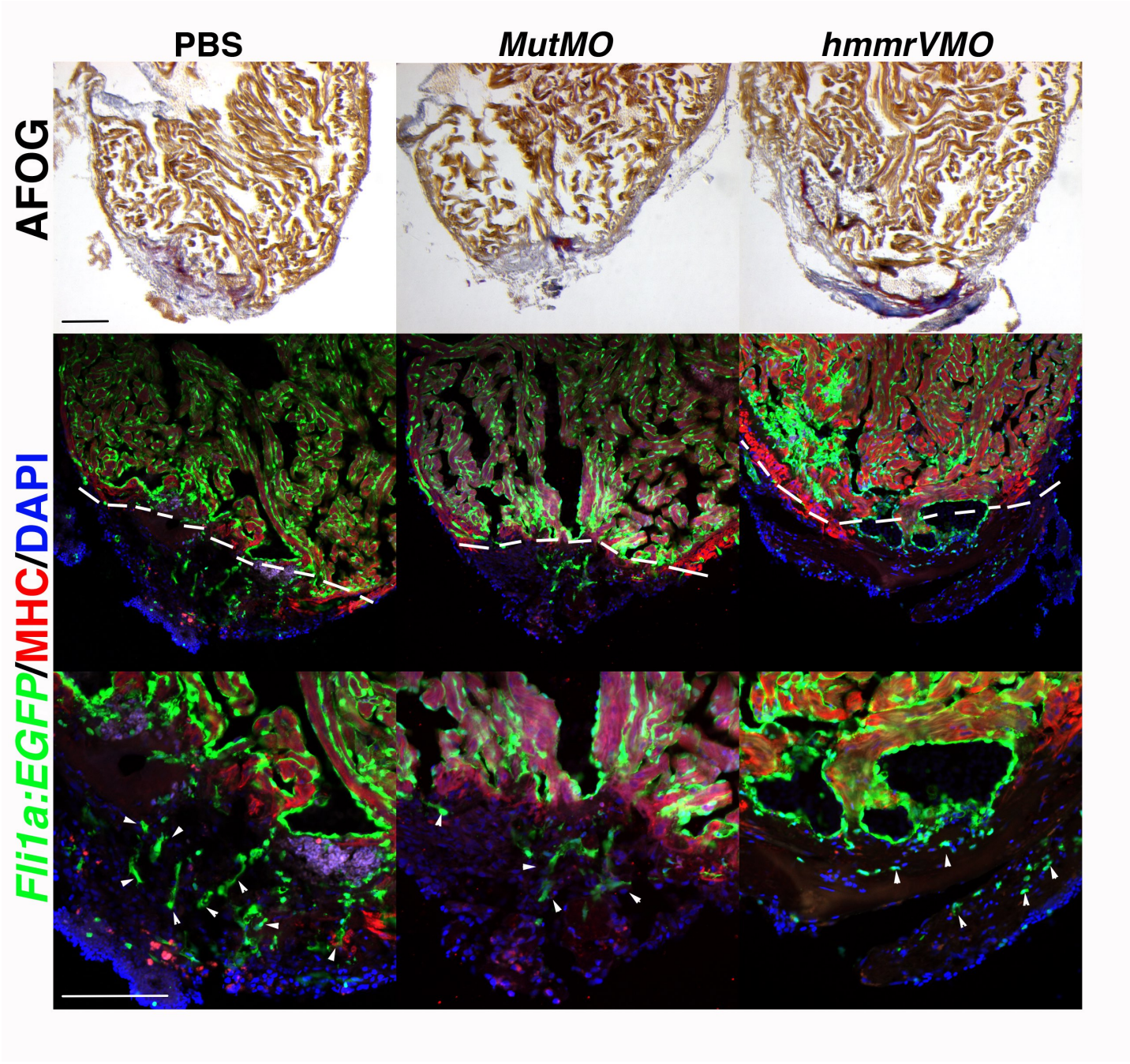

Fig. S8. Hmmr is necessary for angiogenesis. AFOG staining of *Tg(fli1a:EGFP)* hearts at 10 dpa injected for 6 days with PBS (**S8A**; n=5), mismatch MO (**S8B**; n=5), or hmmr-VMO (**S8C**; n=5). Sections are stained with MHC to delineate resection plane. White dashed linees delineate amputation plane. Fish injected with *hmmr-VMO* have decreased vessels formed in the injured area compared to controls. Scale bars, 100 μm.

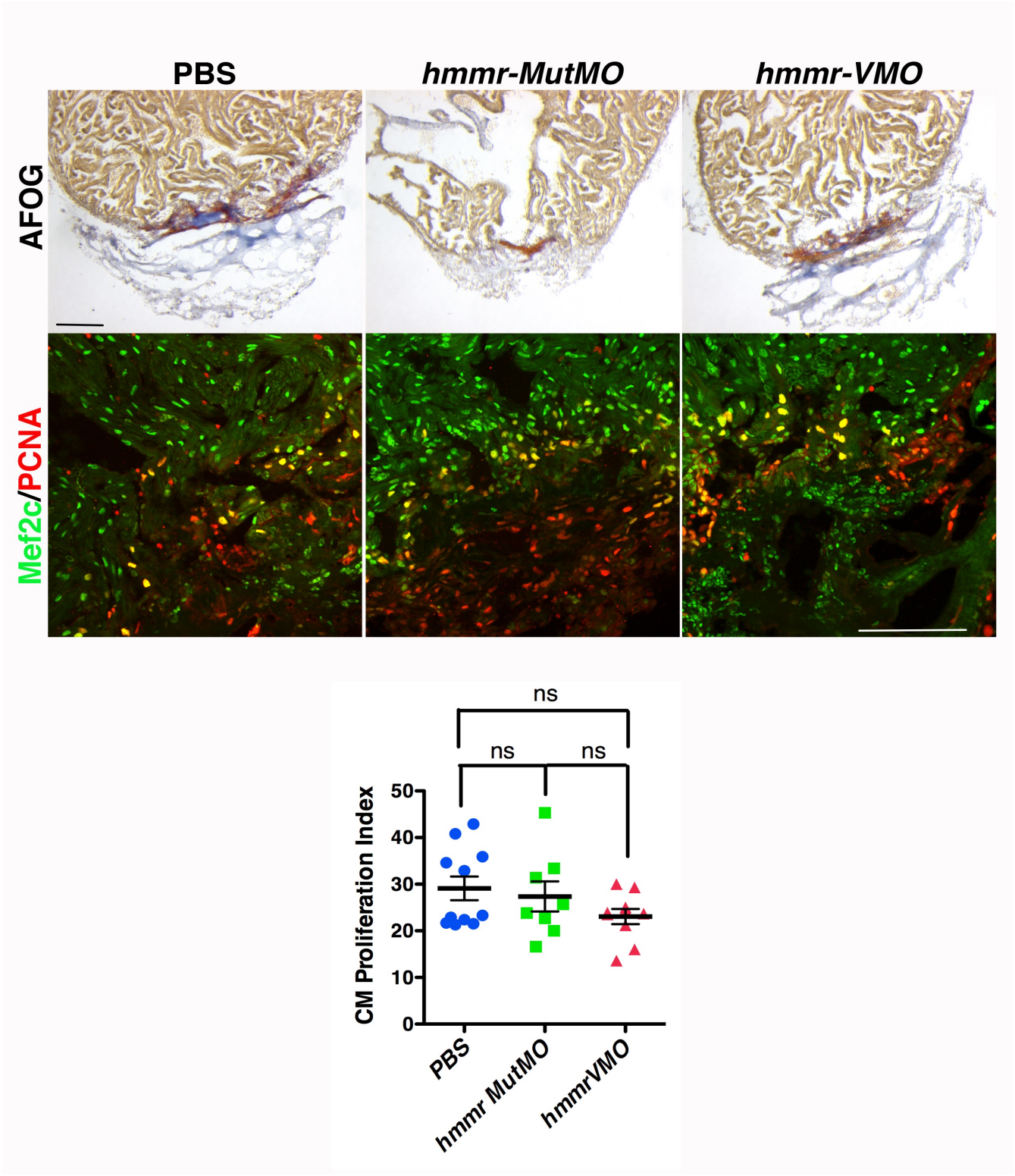

Fig. S9. Cardiomyocyte proliferation is not affected by *hmmr* knockdown. AFOG staining of hearts at 7 dpa injected for 6 days with PBS (n=11), *mutant VMO* (n=8), and *hmmr-VMO* (n=8) and relative PCNA and Mef2c immunostaining to show cardiomyocyte proliferation. Graph showing that the proliferation index was unchanged in PBS, *mutant VMO*, and *hmmr-VMO* injected hearts. Black bars in graphs indicate the mean and error bars the SEM. Data were not statistically different between the treatments. Scale bars, 100 μm. ns, not significant.

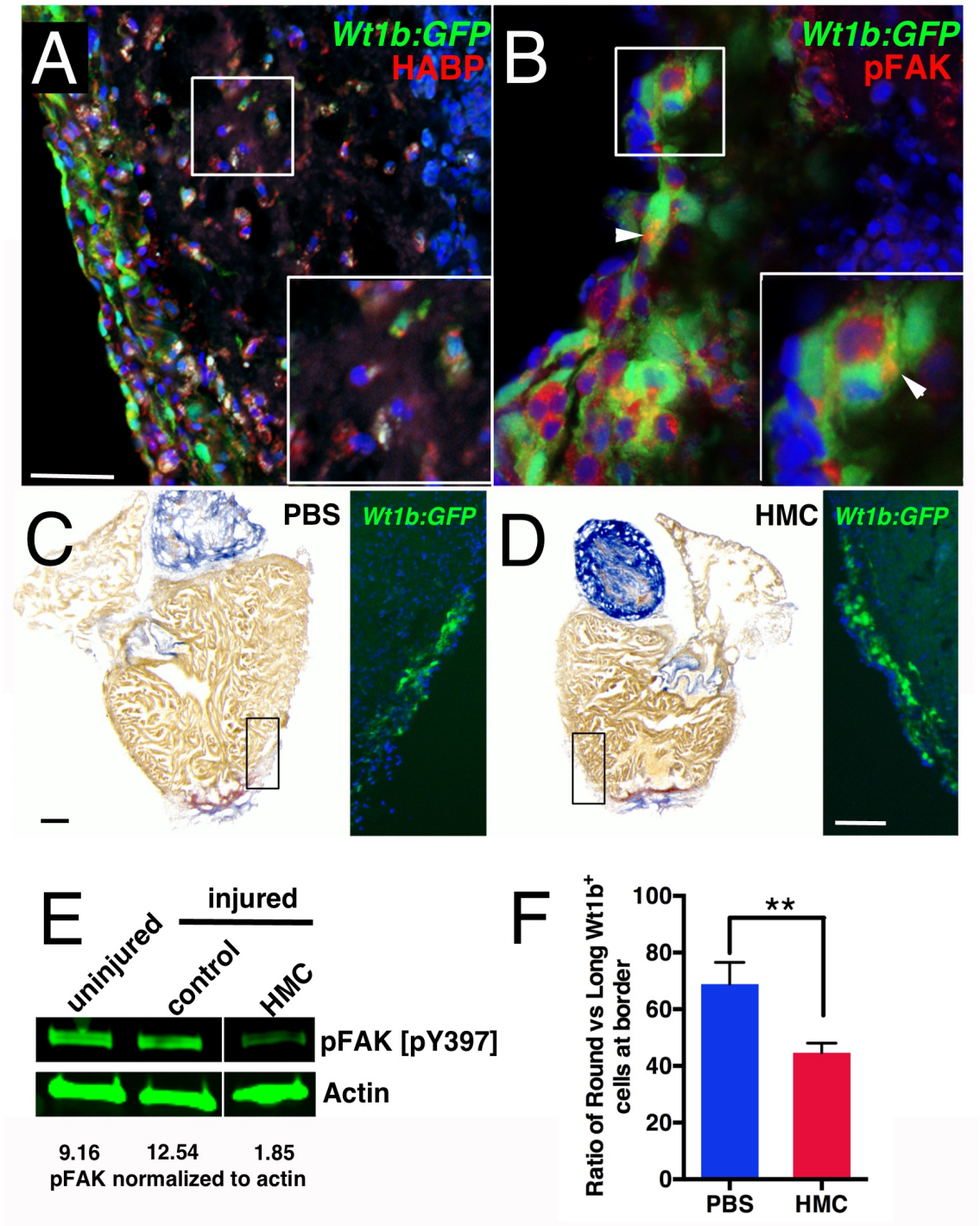

Fig. S10. HA and pFAK detection in epicardial cells. (**S10A**) Confocal pictures of *Tg(wt1b:EGFP)* heart at 7 dpa stained for HA using biotin labeled HABP. (**S10B**) *wt1b:EGFP* cells are pFAK positive. White boxes indicate the area with higher magnification. (**S10C, S10D**) AFOG staining of *Tg(wt1b:EGFP)* and corresponding fluorescent image of hearts at 5 dpa injected with PBS (**S10C**; n=2) and HMC (**S10D**; n=2) showing that activation of epicardial cells are unalterated after HMC injections. Black boxes show the area where the confocal picture was taken. Scale bars, 50 μm (A, B), 200 μm (C, D). (**S10E**) Western blot of pFAK in uninjured hearts and hearts at 1 dpa, after 6 hours of injections with PBS or HMC. Actin was used as loading control. pFAK is reduced after injection of HMC. Bands normalization values are derived from one representative experiment. (**S10F**) Graph showing the ratio of round vs long epicardial cells after injection of HMC, compared to control PBS.

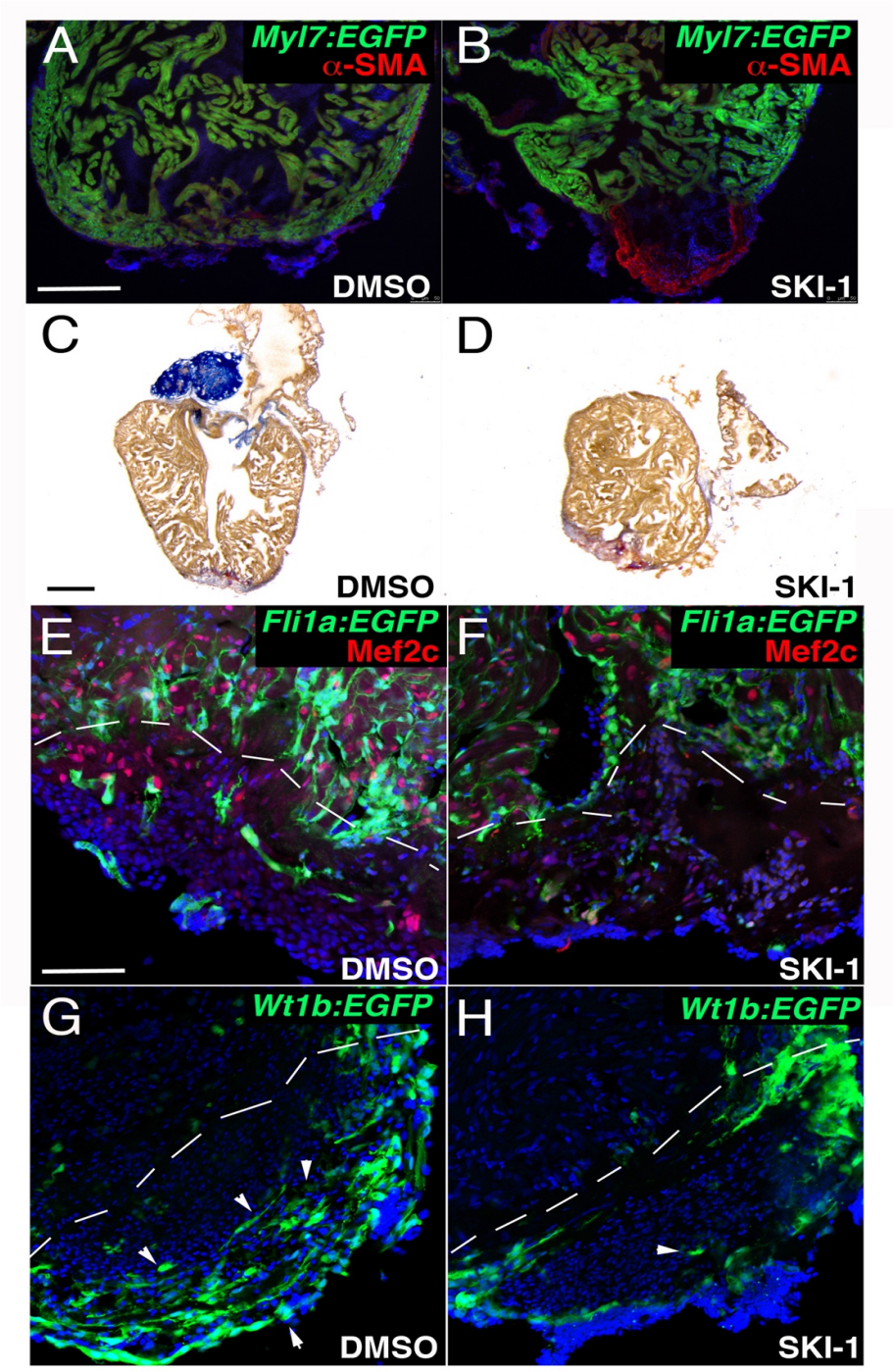

Fig. S11. Src kinase inhibition impaired cardiac regeneration, reducing angiogenesis and epicardial cell migration. (**S11A-S11B**) *Tg(myl7:EGFP)* hearts at 30 dpa immunostained for α-SMA (red), a marker for myofibroblasts. Hearts injected with SKI-1 (**S11B**; n=13), a Src Kinase Inhibitor, showed a clot composed of myofibroblasts, while hearts injected with DMSO (**S11A**; n=14) regenerated properly and had small or no deposition of myofibroblasts (**A**). (**S11C-S11F**) SKI-1 injections suppressed angiogenesis. AFOG staining of hearts at 10 dpa, injected for 6 days with DMSO (**S11C**; n=8), or SKI-1 (**S11D**; n=7) and relative confocal pictures of *Tg(fli1a:EGFP)* hearts injected with DMSO (**S11E**) or SKI-1 (**S11F**), immunostained for Mef2c (red). (**S11G-S11H**) Epicardial cell migration is reduced after SKI-1 injections. *Tg(wt1b:EGFP)* hearts at 7dpa , injected with DMSO (**S11G**; n=5) or with SKI-1 (**S11H**; n=5). DAPI is shown in blue. Scale bars, 100 μm.

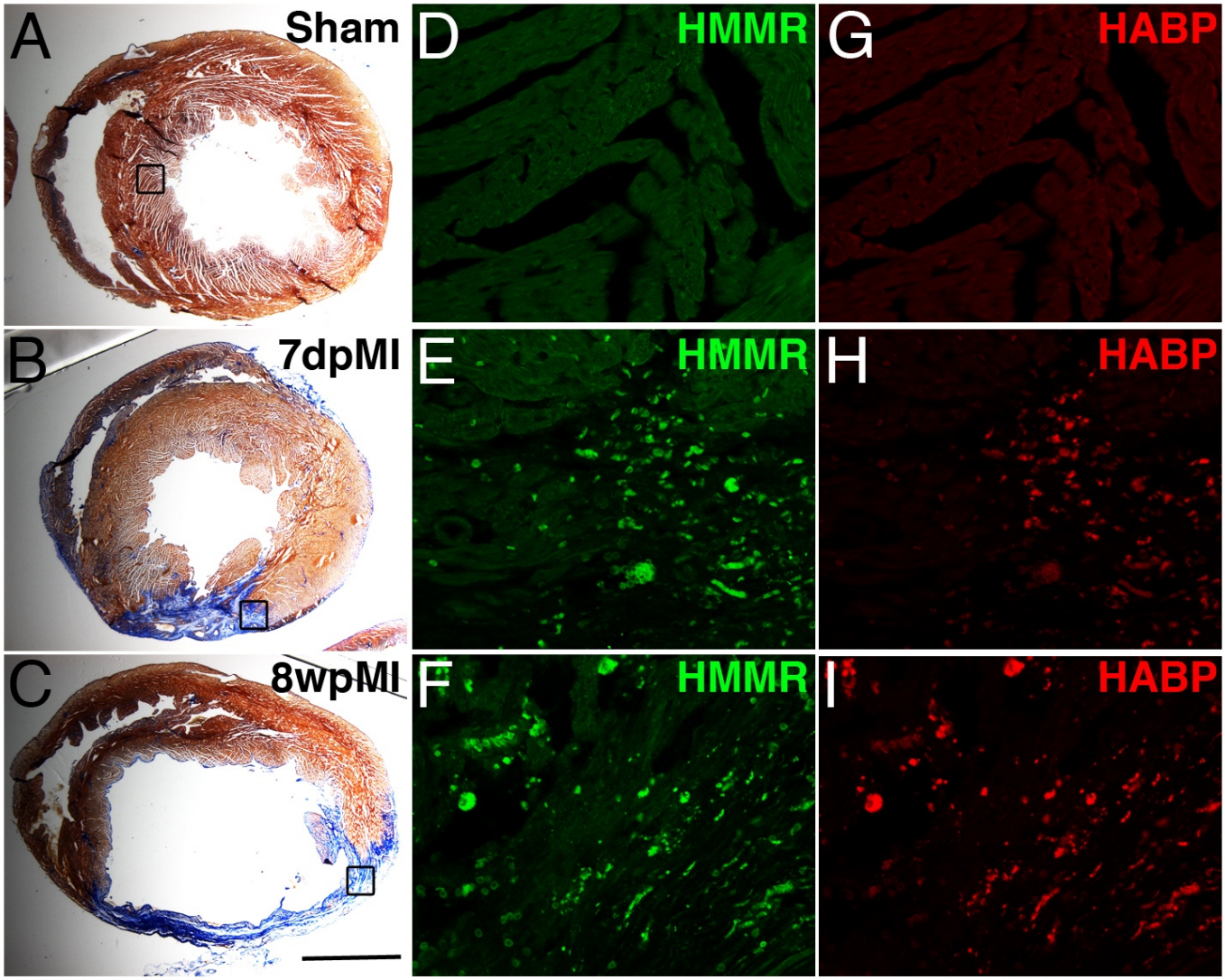

Fig. S12. Detection of HMMR and HA in rat heart scar tissue after MI. (**S12A-S12C**) AFOG staining of rat hearts sham operated (**S12A**; n=2), 7 days post MI (dpMI) (n=4) (**S12B**; n=4), and 8 weeks post MI (wpMI) (**S12C**; n=4). Immunostaining for HMMR (green) revealed expression in the scar tissue (**S12E, S12F**) but not in sham operated rats (**S12D**). (**S12G-S12I**) HA (red) was detected in the scar tissue following cardiac injury (**S12H, S12I**). Black boxes show the area where the confocal picture was taken. Scale bars: 500 µm (A to C), 100 µm (D to I).